THE PROTEIN TYROSINE KINASE SUBSTRATE LAT AND ITS USE IN THE INDENTIFICATION OF (ANT)AGONISTS OF THE KINASE

This invention was made with government support from a grant from the National Institute of Child Health and Human Development (NICHHD). The United States Government has certain rights in the invention.

FIELD OF THE INVENTION

The invention generally relates to compositions and methods for identifying and testing tyrosine kinase signaling pathway agonists and antagonists, and more particularly, methods and compositions for screening compounds and identifying compounds that will modulate the interaction of protein tyrosine kinase substrates with their intracellular ligands, as well as between their intracellular ligands and other members of the signaling pathway.

BACKGROUND OF INVENTION

The immune system has evolved to provide an organism with a flexible and dynamic mechanism to respond specifically to a wide variety of antigenic insults. In order for an immune response to occur following antigenic challenge, the antigen must not only be recognized by antigen specific lymphocytes, but this recognition event must lead to a variety of cellular responses. T lymphocytes, together with B lymphocytes, represent the two antigen specific components of the cellular immune system. T cells are central to the immune response and control the response to infectious agents, tumors and transplants.

The T cell receptor for antigen (TCR) recognizes and binds foreign antigens. This binding event leads to T cell activation. The activation of T lymphocytes is a complex process which results in cell growth and differentiation. The engagement of the TCR on mature peripheral T cells initiates multiple intracellular signals that can lead to cellular proliferation and the acquisition of complex effector functions.

The biochemical mechanisms that couple receptor binding to these intracellular events have been intensively investigated. Early events such as activation of tyrosine phosphorylation, elevation of intracellular calcium, activation of lipid-dependent kinases, and activation of Ras and its downstream kinase cascade are well known [Weiss, A. and Littman, D. R., Signal transduction by lymphocyte antigen receptors, *Cell* 76:263-274 (1994); Cantrell,

D., T Cell Antigen Receptor Signal Transduction Pathways, *Annu. Rev. Immunol.*, 14:259-274 (1996)]. Moreover, there has been analysis of the events involved in transcriptional induction of T cell-specific lymphokines. There remain, however, significant gaps in the understanding of TCR signaling, particularly in how the early tyrosine phosphorylation events couple receptor activation to later cellular events.

Perhaps the most critical insight of the past decade in the study of signal transduction has been the recognition that activation of receptor tyrosine kinases results in the assembly of multimolecular complexes at the cytoplasmic domain of the receptor. Likewise, a major role of the tyrosine phosphorylation cascade initiated by TCR engagement is the assembly of multimolecular signaling complexes at and near the TCR itself. The identity and functions of these proteins that engage in such molecular interactions, are critical in the understanding of T cell receptor signal transduction.

SUMMARY OF THE INVENTION

The invention generally relates to compositions and methods for identifying and testing tyrosine kinase signaling pathway agonists and antagonists, and more particularly, methods and compositions for screening compounds and identifying compounds that will modulate the interaction of protein tyrosine kinase substrates with their intracellular ligands, as well as between their intracellular ligands and other members of the signaling pathway. It is not intended that the present invention be limited to particular signaling pathways. The present invention contemplates that the methods and compositions described herein will be useful for identifying protein tyrosine kinase (PTK) downstream signaling proteins, particularly in cells including but not limited to T cells, NK cells, and mast cells, and will enable the identification of compounds that will modulate the interactions of protein tyrosine kinase that bind particular intracellular ligands, as well as between their intracellular ligands and other members of the signaling pathway.

In preferred embodiments, "Linker for Activation of T-cells" (LAT, and in particular, fragments of LAT) are useful in drug screening assays designed to identify drugs that interfere with the specific binding of Grb2 or GST-GRb2 with LAT and thereby block the activation of downstream signaling molecules.

In one embodiment, the present invention contemplates screening compounds and identifying compounds that modulate the interactions of T cell protein kinases, and their substrates, in particular ZAP-70 and/or Syk tyrosine kinase.

Furthermore, the present invention contemplates identifying ZAP-70 and/or Syk substrates and ZAP-70 and/or Syk-binding ligands, and compounds that will modulate the interaction of ZAP-70 and/or Syk kinase with ZAP-70 and/or Syk substrates and ZAP-70 and/or Syk- binding ligands.

In one embodiment, the present invention contemplates identifying compounds that modulate the interaction of LAT, which binds to activated, Tyr phosphorylated ZAP-70 and/or Syk kinases.

In preferred embodiments, LAT (and, in addition, fragments of LAT and mutated LAT) are useful in drug screening assays designed to identify drugs that interfere with the specific binding activated ZAP-70 and/or Syk kinases with their substrates and thereby block the activation of downstream signaling molecules.

In other embodiments, the present invention contemplates identifying compounds that modulate the interaction of LAT, which may bind to kinases other than ZAP-70 and/or Syk. In other embodiments, the invention provides an isolated LAT polypeptide, or a fragment thereof, having ZAP-70 and/or Syk kinase-specific binding affinity. The invention provides nucleic acids encoding the LAT polypeptide and LAT fragments as part of expression vectors for introduction into cells. The invention provides methods of identifying intracellular molecules which interact with LAT or LAT fragments, as well as exogenous agents (i.e. drugs) which disrupt or enhance the binding of LAT and/or fragments thereof to such intracellular targets.

The claimed polypeptide LAT finds particular use in screening assays for agents or lead compounds for agents useful in the diagnosis, prognosis or treatment of disease, particularly disease associated with undesirable cell growth, differentiation, proliferation and T cell anergy. One such assay involves forming mixtures of 1) LAT (or fragments thereof) and 2) an intracellular LAT-binding ligand, in the presence or absence of 3) a prospective drug candidate. The mixtures are made under conditions that permit the binding of the intracellular LAT-binding ligand to LAT (or fragments thereof) and the mixtures are then analyzed for the presence of such binding. A difference in such binding in the presence of such a drug candidate indicates that the agent is capable of modulating the binding of LAT (or fragments thereof) to an intracellular LAT-binding ligand.

It is not intended that the present invention be limited by the species (human, murine, rat, etc.) of the binding ligands described above. The polypeptide LAT and LAT fragments may bind across species. Moreover, the nucleic acid sequences described herein allow for

the identification of homologues in other species by various methods, including but not limited to amplification (e.g. PCR) using primers designed from the nucleic acid sequence of one species (e.g. mouse) on the nucleic acid template of another species (e.g. human).

In one embodiment, the present invention contemplates an isolated nucleic acid encoding at least a fragment of a protein having the amino acid sequence set forth in SEQ ID NO:4. It is not intended that the present invention be limited by the size or nature of the fragment (although it is preferred that such fragments are capable of binding kinases). In one embodiment, said nucleic acid comprises SEQ ID NO:1 and encodes full-length LAT as set forth in (SEQ ID NO:4). In yet another embodiment, said nucleic acid encodes a fusion protein.

It is not intended that the present invention be limited as to the specific nature of the nucleic acid encoding the peptides described above. In one embodiment, said nucleic acid is contained in a vector. In another embodiment, said vector is in a host cell.

The present invention also contemplates complexes of ligands. In one embodiment, the present invention contemplates a composition, comprising a LAT -kinase complex comprising a purified peptide having at least a portion of the amino acid sequence set forth in SEQ ID NO:4 specifically bound to activated ZAP-70 and/or Syk (or other kinases). Again, the peptides bound specifically to activated ZAP-70 and/or Syk kinases may be full-length LAT or a fragment defined by a portion of the amino acid sequence as set forth in SEQ ID NO:4. The peptide may be part of a fusion protein. The complex can also contain other ligands, such as effector molecules downstream to PTKs. The complexes can be used to identify other ligands (as described below).

As noted above, the present invention contemplates compound screening assays. In one embodiment, the present invention contemplates a method for compound screening, comprising: a) providing: i) a peptide comprising at least a portion of the amino acid sequence set forth in SEQ ID NO:4, wherein said portion is capable of binding to ZAP-70 and/or Syk kinases, ii) ZAP-70 and/or Syk kinases, and iii) one or more compounds for screening; b) mixing, in any order, said peptide, said ZAP-70 and/or Syk kinases and said one or more compound; and c) measuring the extent of binding of said peptide to said ZAP-70 and/or Syk kinases. Again, the peptides may be full-length LAT or mutated LAT or a fragment defined by a portion of the amino acid sequence as set forth in SEQ ID NO:4. The peptide may also be part of a fusion protein. The present invention also contemplates embodiments where either the peptide or kinase is bound to other ligands, such as effector

molecules downstream to PTKs or other PTKs. These complexes can be used in the compound screening assay described above.

The present invention specifically contemplates an isolated polypeptide comprising at least a portion of the amino acid sequence of SEQ ID NO:4. It is not intended that the present invention be limited to a specific portion. However, in one embodiment, said portion comprises a region comprising at least one tyrosine. In another embodiment, said portion comprises more than one tyrosine (and more preferably more than five tyrosine residues). The present invention also contemplates domains of the LAT protein. While not limited to any particular domain, domains such as the cytosolic domain (defined approximately by amino acids 28 to 233).

The present invention also specifically contemplates antibodies (both polyclonal and monoclonal) to LAT, LAT fragments, mutant LAT, mutant LAT fragments, and LAT/LAT Binding Ligand complexes. In one embodiment, the present invention contemplates A purified antibody which binds specifically to a polypeptide comprising at least a portion of the amino acid sequence of SEQ ID NO:4.

The present invention also contemplates an isolated polynucleotide encoding the polypeptide comprising the sequence of SEQ ID NO:4. In one embodiment, said polynucleotide comprises the sequence of SEQ ID NO: 1. The present invention contemplates embodiments where said polynucleotide is contained on a recombinant expression vector and where said expression vector containing said polynucleotide sequence is contained within a host cell.

In some embodiments, the present invention contemplates nucleic acids capable of hybridizing to portions of the LAT gene. In one embodiment, the present invention contemplates a polynucleotide sequence that hybridizes under stringent conditions to the nucleic acid sequence of SEQ ID NO:1.

As discussed above, screening methods are contemplated. In a preferred embodiment, the present invention contemplates a method of screening a compound, said method comprising: a) providing, in any order: i) a peptide comprising at least a portion of the amino acid sequence set forth in SEQ ID NO:4, wherein said portion is capable of binding to a LAT binding ligand; ii) a LAT binding ligand; and iii) one or more compounds for screening; b) mixing, in any order, said peptide, said LAT binding ligand and said one or more compound; and c) measuring the extent of binding of said peptide to said LAT binding ligand.

It is not intended that the present invention be limited to a particular LAT binding ligand. In one embodiment, said LAT binding ligand comprises a tyrosine kinase. In another embodiment, said kinase comprises ZAP-70 kinase. In yet another embodiment, said kinase comprises Syk kinase.

In the embodiments described above, it may be useful to use the entire LAT polypeptide or fragments. In addition, it may be useful where said peptide is part of a fusion protein.

The present invention also contemplates detecting LAT (e.g. detecting LAT in cells and other biological samples). In one embodiment, the present invention contemplates a method for detecting the presence of a portion of the polypeptide having the amino acid sequence set forth in SEQ ID NO:4, said method comprising the steps of: a) providing in any order: i) an antibody capable of reacting with a portion of the polypeptide having the sequence set forth in SEQ ID NO:4; and ii) a sample suspected of containing at least a portion of the polypeptide having the sequence set forth in SEQ ID NO: 4; b) combining said antibody and said sample under conditions such that a complex is formed between said antibody and said portion of said polynucleotide; and c) detecting said complex. Again, the antibody can be polyclonal antibody or monoclonal antibody. A variety of cells and samples are contemplated, including but not limited to NK cells, mast cells and lymphocytes.

The present invention also contemplates using antibodies in other formats. In one embodiment, LAT is detected on gels by immunoblotting.

The present invention is not limited to antibody detection. It may be useful to detect nucleic acid encoding the LAT polypeptide or detect gene expression. In one embodiment, the present invention contemplates a method for detecting the presence of polynucleotide sequences encoding at least a portion of LAT gene in a sample, said method comprising the steps of: a) providing in any order: i) a polynucleotide comprising a sequence that hybridizes under stringent conditions to the nucleic acid sequence of SEQ ID NO:1; and ii) a sample suspected of containing nucleic acid comprising the sequence of SEQ ID NO:1; b) combining said polynucleotide and said sample under conditions such that a hybridization complex is formed between said polynucleotide and said sample nucleic acid; and c) detecting said hybridization complex. The present invention contemplates detecting both RNA and DNA. Again, a variety of cells and samples can be used, including but not limited to, NK cells, mast cells and lymphocytes. It is desirable that said detected hybridization complex correlates with expression of the LAT gene in said lymphocytes.

DESCRIPTION OF FIGURES

FIGURE 1 shows the purification, cDNA cloning, and the deduced amino acid sequence of LAT (Linker for activation of T cells)

FIG. 1(A) is a representative immunoblot of Jurkat cells stimulated with C305 (+) or unstimulated cells (-), analyzed by immunoblotting with anti-phosphotyrosine antibody (PY20).

FIG. 1(B) shows the resolution by reverse-phase microbore HPLC of the affinity-purified p36-38 protein (isolated from stimulated Jurkat cells). Five peptide peaks (fraction 31, 40, 48, 55, 82) were selected for mass spectrometry and microsequencing.

FIG. 1(C) shows the analysis of peptides from the indicated peaks. The single amino acid code is used and with the following additional: X=no assignment, uppercase=full confidence assignment, lowercase=ambiguous residue and n.d.=not determined.

FIGURE 2 shows the characterization of the LAT protein.

FIG. 2(A) is a representative immunoblot of 293T cells transfected with empty vector (pcDNA3) or with myc-tagged LAT in pcDNA3 (+). Post-nuclear lysates of 293T cells were immunoprecipitated with anti-myc antibody (9E10), resolved on SDS-PAGE and immunoblotted with anti-myc antibody.

FIG. 2(B) is a representative immunoblot of lysates from C305 stimulated Jurkat T cells. Proteins immunoprecipitated with rabbit anti-LAT antibody, were treated with calf intestine alkaline phosphatase where indicated, and blotted with anti-phosphotyrosine (pY) and anti-LAT antibodies.

FIG. 2(C) depicts the kinetics of LAT phosphorylation in OKT3 stimulated Jurkat cells. Lysates were immunoprecipitated with anti-LAT antibody, immunoprecipitated proteins were resolved on SDS-PAGE, and blotted with anti-phosphotyrosine (pY) and anti-LAT antibodies.

FIG. 3. shows the tissue distribution of LAT.

FIG. 3(A) is a representative Northern blot analysis of poly $(A)^+$ RNA from different human tissues.

FIG. 3(B) depicts the Northern blot analysis of total RNA from different cell lines: Jurkat (T cell), YT (NK-like cell), THP1 (monocyte), Raji (B lymphoma), Jiyoye (B lymphoma), K562 (myelomonocytic cells), HeLa (fibroblastoid) and RBL (mast cell). The same membrane was also hybridized with a β-actin probe.

FIGURE 4 shows LAT is phosphorylated by PTKs and associates with Grb2, Grap, the p85 subunit of PI-3K and PLC-γ1 in 293T cells.

FIG. 4(A) is a representative immunoblot showing tyrosine phosphorylation of LAT by Syk and ZAP-70 PTKs. FLAG-tagged LAT in pcDNA3 was transfected in 293T cells either alone or cotransfected with other PTKs as indicated.

FIG. 4(B) is a representative immunoblot, showing association of LAT with signaling molecules in 293T cells. FLAG-tagged LAT was cotransfected with pCEFL/HA-Grb2, pSRα/Grap-myc, pcDNA3/HA-p85 and pcDNA3/PLC-γ1 in the absence or presence of ZAP-70 and Lck PTKs. Brij lysates of transfected 293T cells were immunoprecipitated with anti-FLAG, and association of LAT with tested proteins was determined by blotting with specific antibodies.

FIG. 4(C) is a representative immunoblot, showing LAT associates with Grb2 or Grap by reciprocal immunoprecipitation. Lck, ZAP-70, and LAT were cotransfected with either Grb2-HA or Grap-myc as indicated, and 293T cell lysates were immunoprecipitated with anti-FLAG and anti-HA antibodies if cotransfected with Grb2-HA, or anti-FLAG and anti-Myc antibodies if cotransfected with Grap-myc.

FIGURE 5 shows LAT associates with Grb2, p85 and PLC-γ1 in Jurkat T cells.

FIG. 5(A) is a representative immunoblot, showing GST-Grb2 associates with LAT through its SH2 domain. GST fusion proteins cross-linked to glutathione-agarose were used to precipitate proteins from unstimulated or stimulated with C305 Jurkat lysates. GST-Grb2 (SH2*) has a mutation in the SH2 domain (R86K). GST-Grb2 (SH3*) has mutations at both N-terminal and C-terminal SH3 domains (P49L/G203R)

FIG. 5(B) is a representative immunoblot, depicting lysates from unstimulated or C305 stimulated Jurkat T cells subjected to anti-Grb2 or anti-PLC-γ1 immunoprecipitation, and immunoblotted with anti-LAT antibody.

FIG. 5(C) is a representative immunoblot, depicting lysates from Jurkat T cells subjected to preimmune serum or anti-LAT antibody immunoprecipitation, resolved on SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (4G10). The same membrane was stripped and also blotted with anti-Grb2, p85, Vav, Cbl, PLC-γ1 to identify specific proteins. Only anti-p85 blotting is shown in this figure.

FIGURE 6 shows overexpression of a mutant form of LAT (Y171/191F) blocks complex formation and TCR mediated AP-1 and NFAT transcriptional activation.

FIG. 6(A) Jurkat cells were stably transfected with wild-type (WT) or mutant (Y171/191F) LAT. Exogenous LAT was immunoprecipitated with anti-myc antibody from cells either left unstimulated or stimulated with C305 (1:50). Samples were analyzed on SDS-PAGE and blotted with 4G10. The same membrane was also blotted with anti-PLC-γ1, anti-p85, anti-Grb2, anti-SOS, and anti-LAT after stripping.

FIG. 6(B). shows secreted alkaline phosphatase (SEAP) activity and the data shown is representative of three independent experiments. Jurkat TAg cells were cotransfected with 20 μg pSX-AP-1/SEAP with 20μg empty vector, pEF/LAT(WT), or pEF/LAT(Y171/191F), and then stimulated with OKT3 (1:1000), or stimulated with PMA and ionomycin, or left unstimulated.

FIG.6(C) shows SEAP activity and the data shown is representative of three independent experiments. Jurkat TAg cells were cotransfected with 20 μg pSX-NFAT/SEAP with 20μg empty vector, pEF/LAT(Wt), or pEF/LAT(Y171/191F), and the same procedure used for stimulation and SEAP assay as in (B).

FIGURE 7A shows the nucleotide sequence (SEQ ID NO:1) of human LAT.

FIGURE 7B shows the nucleotide sequence (SEQ ID NO: 2) of an alternative splice variant of human LAT.

FIGURE 7C shows the nucleotide sequence (SEQ ID NO: 3) of murine LAT.

FIGURE 7D shows the amino acid sequence (SEQ ID NO:4) of human LAT and of murine LAT (SEQ ID NO:5).

FIGURE 8A shows the nucleotide sequence (SEQ ID NO:6) of human ZAP-70 kinase.

FIGURE 8B shows the amino acid sequence (SEQ ID NO:7) of human ZAP-70 kinase.

FIGURE 9A shows the nucleotide sequence (SEQ ID NO:8) of human Syk kinase.

FIGURE 9B shows the amino acid sequence (SEQ ID NO:9) of human Syk kinase.

FIGURE 10A shows the nucleotide sequence (SEQ ID NO:10) of human Grb2.

FIGURE 10B shows the amino acid sequence (SEQ ID NO:11) of human Grb2.

FIGURE 11A shows the nucleotide sequence (SEQ ID NO:12) of human Vav.

FIGURE 11B shows the amino acid sequence (SEQ ID NO:13) of human Vav.

FIGURE 12A shows the nucleotide sequence (SEQ ID NO:14) of human Cbl.

FIGURE 12B shows the amino acid sequence (SEQ ID NO:15) of human Cbl.

FIGURE 13A shows fractions from a sucrose gradient separating a 1% Triton lysate of unstimulated Jurkat cells. Resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (4G10).

FIGURE 13B shows fractions from a sucrose gradient separating a 1% Triton lysate of OKT3-stimulated Jurkat cells. Analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody.

FIGURE 13C shows the same membrane from (A) and (B). Was stripped and blotted with anti-PCL-γ1, CBl, Vav, ZAP-70, Lck and Grb2 antibodies.

FIGURE 14 shows metabolic labeling of Wt and mutant forms of LAT with [^{3}H]palmitate.

FIGURE 15 shows localization of Wt and mutant LAT to the plasma membrane and glycolipid-enriched microdomains.

FIGURE 16 shows tyrosine phosphorylation of Wt and mutant LAT.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The abbreviations used herein are: LAT, Linker for activation of T cells, mLAT, mutant LAT (Y171/191F); PTKs, Protein tyrosine kinases; PTPS, protein tyrosine phosphatases; GST, glutathione S-transferase; TCR, T cell receptor, BCR, B cell antigen receptor; SEAP, secreted alkaline phosphatase; Tyr, tyrosine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; ITAMs, immunoreceptor tyrosine-based activation motifs; SLP-76, SH2-domain Leukocyte Protein; PLC-γ1, Phospholipase C-gamma1; PI3-K, phosphatidylinositol 3-kinase; IgR, Immunoglobulin receptor; SH2(C), C terminal of Src-homology 2 domain.

The terms "Lck and Fyn" refer to the protein tyrosine kinases of the src family, shown to be involved in TCR signaling.

The terms "Zap-70 and Syk" refer to the protein tyrosine kinases of the syk family, shown to be involved in TCR signaling.

The term "Vav" refers to the human oncogene derived from a locus ubiquitously expressed in hematopoietic cells.

The term "Cbl" refers to the human proto-oncogene c-cbl.

The term "Gbr2" refers to the human epidermal growth factor receptor-binding protein.

The term "adaptor" or "linker protein" refers to a protein or scaffold upon which other signaling molecules assemble, for e.g. the Shc protein, Grb2, ZAP-70 etc

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor thereof. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired enzymatic activity is retained.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications (e.g. deletions, substitutions, etc.) in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "transfection" as used herein refers to the introduction of a transgene into a cell. The term "transgene" as used herein refers to any nucleic acid sequence which is introduced into the genome of a cell by experimental manipulations. A transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (*i.e.*, "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, etc.) relative to the naturally-occurring sequence. The term "heterologous DNA sequence" refers to a nucleotide sequence which is not endogenous to the cell into which it is introduced. Heterologous DNA includes a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA also includes a nucleotide sequence which is naturally found in the cell into which it is introduced and which contains some modification relative to the naturally-occurring sequence. Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is introduced. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, DNA sequences which encode selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.* Yet another example of a heterologous

DNA includes a nucleotide sequence which encodes a ribozyme which is found in the cell into which it is introduced, and which is ligated to a promoter sequence to which it is not naturally ligated in that cell.

Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, biolistics (*i.e.*, particle bombardment) and the like.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of a transgene into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated one or more transgenes into the genomic DNA.

The term "transient transfection" or "transiently transfected" refers to the introduction of one or more transgenes into a transfected cell in the absence of integration of the transgene into the host cell's genome. The term "transient transfectant" refers to a cell which has transiently integrated one or more transgenes.

A "transgenic organism" as used herein refers to an organism in which one or more cells has been transiently transfected or stably transfected with a transgene by experimental manipulation. Trangenic organisms may be produced by several methods including the introduction of a "transgene" comprising nucleic acid (usually DNA) into an embryonic target cell or a somatic target cell of a non-human organism by way of human intervention.

The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, usually more than three (3), and typically more than ten (10) and up to one hundred (100) or more (although preferably between twenty and thirty). The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, or a combination thereof.

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid

sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends.

When two different, non-overlapping oligonucleotides anneal to different regions of the same linear complementary nucleic acid sequence, and the 3' end of one oligonucleotide points towards the 5' end of the other, the former may be called the "upstream" oligonucleotide and the latter the "downstream" oligonucleotide.

The term "primer" refers to an oligonucleotide which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. An oligonucleotide "primer" may occur naturally, as in a purified restriction digest or may be produced synthetically.

A primer is selected to be "substantially" complementary to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

"Hybridization" methods involve the annealing of a complementary sequence to the target nucleic acid (the sequence to be detected). The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, *Proc. Natl. Acad. Sci. USA* 46:453 (1960) and Doty *et al.*, *Proc. Natl. Acad. Sci. USA* 46:461 (1960) have been followed by the refinement of this process into an essential tool of modern biology. Nonetheless, a number of problems have prevented the wide scale use of hybridization as a tool in human diagnostics. Among the more formidable problems are: 1) the inefficiency of hybridization; 2) the low concentration of specific target sequences in a mixture of genomic DNA; and 3) the hybridization of only partially complementary probes and targets.

With regard to efficiency, it is experimentally observed that only a fraction of the possible number of probe-target complexes are formed in a hybridization reaction. This is particularly true with short oligonucleotide probes (less than 100 bases in length). There are

three fundamental causes: a) hybridization cannot occur because of secondary and tertiary structure interactions; b) strands of DNA containing the target sequence have rehybridized (reannealed) to their complementary strand; and c) some target molecules are prevented from hybridization when they are used in hybridization formats that immobilize the target nucleic acids to a solid surface.

Even where the sequence of a probe is completely complementary to the sequence of the target, *i.e.*, the target's primary structure, the target sequence must be made accessible to the probe via rearrangements of higher-order structure. These higher-order structural rearrangements may concern either the secondary structure or tertiary structure of the molecule. Secondary structure is determined by intramolecular bonding. In the case of DNA or RNA targets this consists of hybridization within a single, continuous strand of bases (as opposed to hybridization between two different strands). Depending on the extent and position of intramolecular bonding, the probe can be displaced from the target sequence preventing hybridization.

Solution hybridization of oligonucleotide probes to denatured double-stranded DNA is further complicated by the fact that the longer complementary target strands can renature or reanneal. Again, hybridized probe is displaced by this process. This results in a low yield of hybridization (low "coverage") relative to the starting concentrations of probe and target.

With regard to low target sequence concentration, the DNA fragment containing the target sequence is usually in relatively low abundance in genomic DNA. This presents great technical difficulties; most conventional methods that use oligonucleotide probes lack the sensitivity necessary to detect hybridization at such low levels.

One attempt at a solution to the target sequence concentration problem is the amplification of the detection signal. Most often this entails placing one or more labels on an oligonucleotide probe. In the case of non-radioactive labels, even the highest affinity reagents have been found to be unsuitable for the detection of single copy genes in genomic DNA with oligonucleotide probes. *See* Wallace *et al.*, *Biochimie* 67:755 (1985). In the case of radioactive oligonucleotide probes, only extremely high specific activities are found to show satisfactory results. *See* Studencki and Wallace, *DNA* 3:1 (1984) and Studencki *et al.*, *Human Genetics* 37:42 (1985).

K.B. Mullis *et al.*, U.S. Patent Nos. 4,683,195 and 4,683,202, hereby incorporated by reference, describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for

amplifying the target sequence (which can be used in conjunction with the present invention to make target molecules) consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. There can be numerous "cycles" to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to by the inventors as the "Polymerase Chain Reaction" (hereinafter PCR). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

The present invention specifically contemplates using primers having a portion of the nucleic acid sequence set forth in SEQ ID NO:1 in a PCR reaction to identify homologues of LAT, as well as the LAT gene in other species. Such primers are preferably less than fifty nucleotides in length (although longer primers can be used if desired).

The present invention also contemplates using probes having a portion of the nucleic acid sequence set forth in SEQ ID NO:1. The term "probe" as used herein refers to a labeled oligonucleotide which forms a duplex structure with a sequence in another nucleic acid, due to complementarity of at least one sequence in the probe with a sequence in the other nucleic acid.

In one embodiment, the present invention contemplates a polynucleotide sequence that hybridizes under stringent conditions to the nucleic acid sequence of SEQ ID NO:1. As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. "Stringency" typically occurs in a range from about T_m-5°C (5°C below the T_m of the probe) to about 20°C to 25°C below T_m. As will be understood by those of skill in the art, a stringent hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences. Conditions of "weak" or "low" stringency are often required with

nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually low between such organisms.

The term "label" as used herein refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g.,.^{3}H), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase), biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance. In others, the label is part of the fusion protein, e.g. Green Fluorescent Protein (GFP).

The terms "nucleic acid substrate" and nucleic acid template" are used herein interchangeably and refer to a nucleic acid molecule which may comprise single- or double-stranded DNA or RNA.

The term "substantially single-stranded" when used in reference to a nucleic acid substrate means that the substrate molecule exists primarily as a single strand of nucleic acid in contrast to a double-stranded substrate which exists as two strands of nucleic acid which are held together by inter-strand base pairing interactions.

The term "sequence variation" as used herein refers to differences in nucleic acid sequence between two nucleic acid templates. For example, a wild-type structural gene and a mutant form of this wild-type structural gene may vary in sequence by the presence of single base substitutions and/or deletions or insertions of one or more nucleotides. These two forms of the structural gene are said to vary in sequence from one another. A second mutant form of the structural gene may exist. This second mutant form is said to vary in sequence from both the wild-type gene and the first mutant form of the gene. It should be noted that, while the invention does not require that a comparison be made between one or more forms of a gene to detect sequence variations, such comparisons are possible using particular hybridization conditions as described in U.S. Patent No. 5,652,096, hereby incorporated by reference.

The term "K_m" as used herein refers to the Michaelis-Menton constant for an enzyme and is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity in an enzyme catalyzed reaction.

The term "nucleotide analog" as used herein refers to modified or non-naturally occurring nucleotides such as 7-deaza purines (*i.e.*, 7-deaza-dATP and 7-deaza-dGTP). Nucleotide analogs include base analogs and comprise modified forms of deoxyribonucleotides as well as ribonucleotides. As used herein the term "nucleotide analog" when used in reference to substrates present in a PCR mixture refers to the use of nucleotides other than dATP, dGTP, dCTP and dTTP; thus, the use of dUTP (a naturally occurring dNTP) in a PCR would comprise the use of a nucleotide analog in the PCR. A PCR product generated using dUTP, 7-deaza-dATP, 7-deaza-dGTP or any other nucleotide analog in the reaction mixture is said to contain nucleotide analogs.

"Oligonucleotide primers matching or complementary to a gene sequence" refers to oligonucleotide primers capable of facilitating the template-dependent synthesis of single or double-stranded nucleic acids. Oligonucleotide primers matching or complementary to a gene sequence may be used in PCRs, RT-PCRs and the like.

A "consensus gene sequence" refers to a gene sequence which is derived by comparison of two or more gene sequences and which describes the nucleotides most often present in a given segment of the genes; the consensus sequence is the canonical sequence.

The term "polymorphic locus" is a locus present in a population which shows variation between members of the population (*i.e.*, the most common allele has a frequency of less than 0.95). In contrast, a "monomorphic locus" is a genetic locus at little or no variations seen between members of the population (generally taken to be a locus at which the most common allele exceeds a frequency of 0.95 in the gene pool of the population).

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to

mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAR" and is complementary to a reference sequence "GTATA".

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length LAT cDNA sequence given in a sequence listing or may comprise a complete gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. (U.S.A.)* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences

over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length LAT sequence.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion as compared to the native protein, but where the remaining amino acid sequence is identical to the corresponding positions in the amino acid sequence deduced from a full-length cDNA sequence (e.g., various fragments of LAT

protein). Fragments typically are at least 4 amino acids long, preferably at least 20 amino acids long, usually at least 50 amino acids long or longer, and span the portion of the polypeptide required for intermolecular binding of LAT.

The term "analog" as used herein refers to polypeptides which are comprised of a segment of at least 25 amino acids that has substantial identity to a portion of the deduced amino acid sequence of LAT. The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents are evaluated for potential activity as antagonists or agonists of LAT by inclusion in screening assays described herein below.

GENERAL DESCRIPTION OF THE INVENTION

The invention generally relates to compositions and methods for identifying and testing tyrosine kinase signaling pathway agonists and antagonists, and more particularly, methods and compositions for screening compounds and identifying compounds that will modulate the interaction of protein tyrosine kinase substrates with their intracellular ligands, as well as between their intracellular ligands and other members of the signaling pathway.

Identifying and developing new pharmaceuticals is a multibillion dollar industry in the U.S. alone. Agonists and antagonists of critical proteins in signal transduction pathways provide a promising class of targets for novel therapeutics directed to human diseases. Urgently needed are efficient methods of identifying pharmacological agents or drugs which are active at critical target points of signal transduction pathways. Methods amenable to automated, cost-effective, high throughput drug screening have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

The present invention contemplates elements of the T cell signal transduction pathway as targets for pharmaceutical intervention in the immune system. Immunosuppression is therapeutically desirable in a wide variety of circumstances including transplantation, allergy and other forms of hypersensitivity, autoimmune diseases, infectious diseases and cancer. Cyclosporin, a widely used drug for effecting immunosuppression, is believed to act by inhibiting a calcineurin, a phosphatase which activates certain transcription factors. However, because of side effects and toxicity, clinical indications of cyclosporin (and the more recently developed FK506) are limited. Accordingly, it is desired to identify agents which

specifically target critical points in the pathway, that will be less toxic than current drugs, such as cyclosporin, which target more widely expressed molecules.

In one embodiment, the invention provides compositions and methods for identifying and testing the syk family of protein tyrosine kinase (ZAP-70 and syk) substrates. In another embodiment, the invention contemplates identifying tyrosine kinase signaling pathway agonists and antagonists, and in particular, compositions comprising LAT which is Tyr phosphorylated by ZAP-70 and/or Syk protein tyrosine kinases leading to recruitment of multiple signaling molecules.

In preferred embodiments, the invention contemplates identifying tyrosine kinase signaling pathway agonists and antagonists, and in particular, compositions comprising LAT and fragments thereof, which bind to Grb2

The description of the invention involves : A) T cell antigen receptor signaling, B) TCR-Associated Protein Tyrosine Kinases and their Substrates, C) Multimolecular Complexes in TCR signaling, D) Tyrosine Phosphatases, E) LAT as a ZAP-70 and/or Syk tyrosine kinase substrate, and F) Uses Of The Invention.

A. T Cell Antigen Receptor Signaling

The complexity of molecular associations involved in TCR signaling begins with the TCR, which is composed of 6 different polypeptide chains thought to be organized into an 8-chain structure. These polypeptides include a ligand binding heterodimer ($\alpha\beta$ or $\gamma\delta$) and the non-polymorphic CD3 ε, γ, δ and TCRζ chains. These non-polymorphic chains are required for receptor assembly, cell-surface expression and signaling. Critical regions of the cytoplasmic domains of these chains are the immunoreceptor tyrosine-based activation motifs (ITAMs), three of which are in each of the TCRζ chains, and one in each of the CD3 chains (Cambier, 1995). Since each TCR can contain a TCRζ dimer and two CD3 dimers ($\varepsilon\delta$, $\varepsilon\gamma$), each TCR can contain a total of ten ITAMs.

It has been well documented that these motifs are necessary and sufficient for coupling the TCR to the intracellular signaling machinery, and function by binding key signaling molecules in resting and activated T cells. Upon TCR activation the tyrosine residues within the ITAMs become phosphorylated, permitting the binding of SH2 domain-containing proteins as a consequence of the ability of SH2 domains to bind specific phosphotyrosine-containing polypeptides. Additional signaling molecules are subsequently

recruited to these newly TCR-associated proteins via SH2 or other modular interaction domains.

While a similar requirement of ITAMs for initiating signaling by the B-cell antigen receptor (BCR) and certain immunoglobulin receptors (IgR) has been shown, the TCR is notable for the sheer number of ITAMs present in a single receptor. Two different explanations for the presence of ten ITAMs within a single receptor have been proposed. The multiple ITAMs may provide the capacity to amplify the signal received by each TCR. Indeed, it has been shown that the intensity of the response to signaling by recombinant ITAM-containing polypeptide chains is dependent on the number of ITAMs present. As the T cell may be required to respond strongly in the presence of a low antigen concentration, this multiplicity of ITAMs is potentially quite important. However, recent studies offer an alternative explanation. A specificity of interaction with different ITAMs has been noted for a number of molecules including ZAP-70, PLCγ1, PI3-K and Shc, raising the possibility that ITAMs are bound differentially [Exley, M., Varticovsky, L., Peter, M., Sancho, J. and Terhorst, C., Association of phosphatidylinositol-3-kinase with a specific sequence of the T cell receptor chain is dependent on T cell activation, *J. Biol. Chem.* 269:15140-15146 (1994); Cambier, J. C. and Johnson, S. A. , Differential binding activity of ARH1/TAM motifs, Immunol. Lett. 44:77-80 (1995); Isakov, N., Wange, R. L., Burgess, W. H., Watts, J. D., Aebersold, R. and Samelson, L. E., ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: the tandem SH2 domains of ZAP-70 bind distinct tyrosine-based activation motifs with varying affinity, *J. Exp. Med.* 181:375-380 (1995); Osman, N., Turner, H., Lucas, S., Reif, K. and Cantrell, D.A., The protein interactions of the immunoglobulin receptor family tyrosine-based activation motifs present in the T cell receptor subunits and the CD3, and chains, *Eur. J. Immunol.* 26:1063-1068 (1996)]. Of course, the multiplicity of ITAMs within the TCR may actually serve both purposes.

Another important function of ITAMs may be to mediate an activation-induced association of TCRζ and CD3ε with the actin cytoskeleton. There is increasing evidence to suggest that in resting T cells a fraction of the TCR is tightly associated with the actin cytoskeleton, and that upon TCR stimulation the portion of TCRζ and CD3ε recoverable in the cytochalasin-disruptable, detergent-insoluble fraction increases [Caplan, S., Zeliger, S., Wang, L. and Baniyash, M., Cell-surface expressed T-cell antigen-receptor chain is associated with the cytoskeleton, *Proc. Natl. Acad. Sci. USA* , 92:4768-4772 (1995); Rozdzial, M. M., Malissen, B. and Finkel, T. H. (1995). Tyrosine-phosphorylated T cell

receptor chain associates with actin cytoskeleton upon activation of mature T lymphocytes, *Immunity*, 3:623-633 (1995)]. This association with the actin cytoskeleton requires the intact C-terminal ITAM of TCRζ. Further investigation is needed to determine how these interactions impinge on TCR recycling, trafficking and signaling, and to assess the role of TCR-cytoskeletal interactions in mediating the changes in cell shape and motility patterns that can accompany T cell activation.

B. TCR-Associated Protein Tyrosine Kinases and their Substrates

Immediately downstream of the TCR in the signaling pathway are the TCR-associated protein tyrosine kinases (PTKs). Two families of PTKs have been shown to be involved in TCR signaling. Lck and Fyn are members of the Src family, while ZAP-70 and Syk make up another PTK family. Characteristics of these enzymes have been extensively reviewed [Bolen, J.B., Nonreceptor tyrosine protein kinases, *Oncogene*, 8:2025-2031 (1993); Peri, K. G. and Veillette, A., Tyrosine protein kinases in T lymphocytes. *Chem. Immunol.* 59:19-39 (1994)]. A primary function of the Src-family kinases is to phosphorylate key tyrosine residues within the ITAMs. An additional function of these kinases includes the phosphorylation and concurrent activation of the ZAP-70 kinase. Other substrates for these enzymes remain to be defined, though some candidates such as the receptor for IP_3 (providing a mechanism for Fyn to directly regulate intracellular Ca^{2+}) have been identified [(Jayaraman *et al.*, Regulation of the Inositol 1,4,5-trisphosphate receptor by tyrosine phosphorylation, *Science* 272:1492-1494 (1996)]. It remains to be determined whether Lck and Fyn have any unique substrates, or whether their roles in signaling are redundant. Genetic experiments in which these enzymes are either deleted or over-expressed suggest unique roles for them during development, but these experiments do not address function in a mature T cell that developed in a normal environment.

The relatively recent discovery of the PTKs ZAP-70 and Syk has led to intense investigation of their function in lymphocytes. Syk is central to BCR and IgR function. Though possibly involved in T cell development and the function of certain subsets of γδ T cells, its importance to TCR signaling in mature T cells has not been demonstrated [Cheng *et al.*, Syk tyrosine kinase required for mouse viability and B-cell development, *Nature* 378:303-306 (1995); Fargnoli *et al.*, Syk mutation in Jurkat E6-derived clones results in lack of p72syk expression, *J Biol. Chem.* 270:26533-26537 (1995); Turner *et al.*, Perinatal

lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk, *Nature* 378:298-302 (1995)]. In contrast, study of patients with ZAP-70 deficiency and mice with a genetically engineered absence of ZAP-70 confirm the critical function of this enzyme for TCR-mediated signaling [Arpaia *et al.*, Defective T cell receptor signaling and CD8+ thymic selection in humans lacking ZAP-70 kinase, *Cell* 76:947-958 (1994); Chan *et al.*, ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency, *Science* 264:1599-1601 (1994); Elder *et al.*, Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase, *Science* 264:1596-1599 (1994); Negishi *et al.*, Essential role for ZAP-70 in both positive and negative selection of thymocytes, *Nature* 376:435-438 (1995)].

Certain factors that regulate the activity of ZAP-70 have been identified. That activation of ZAP-70 requires a Src family kinase could be inferred from the initial description of this enzyme [Chan *et al.*, ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR chain, *Cell* 71:649-662 (1992)]. Full enzymatic activity of ZAP-70 expressed in COS cells required co-expression of Lck or Fyn. These results, and the generally recognized ability of tyrosine phosphorylation to regulate PTK activity, lead to an investigation of the sites of tyrosine phosphorylation within activated ZAP-70. Recombinant ZAP-70, when analyzed *in vitro*, becomes tyrosine phosphorylated at a low level at Y126 and Y292 [Watts *et al.*, Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70, *J. Biol. Chem.* 269:29520-29529 (1994)]. However, only when assayed with added recombinant Lck does maximal tyrosine phosphorylation and maximal activity of the enzyme occur. Prominent sites of phosphorylation under these conditions are the adjacent tyrosines 492 and 493 in the kinase domain as well as tyrosines 69, 126, 178 and 292. A similar experiment using kinase-dead ZAP-70 (K369R) and the isolated kinase domain of Lck found only a single prominent site of phosphorylation in ZAP-70 at Y493 [Guanghui *et al.*, Distinct tyrosine phosphorylation sites within ZAP-70 mediate activation and negative regulation of antigen receptor function, *Mol. Cell. Biol.* (1996) In press]. ZAP-70 isolated from intact, activated T cells contains phosphate on Y492 and Y493 as well as on Y292, which lies between the second SH2 domain and the kinase domain (Watts *et al.*, Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70, *J. Biol. Chem.*

269:29520-29529 (1994)]. Another critical observation was that isolated proteolytic phosphopeptides purified from activated ZAP-70 contained phosphate on Y493 alone or on both Y492 and Y493, but not on Y492 alone, suggesting that phosphorylation at Y493 precedes and is required for phosphorylation of Y492 (Chan *et al.*, Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function, *EMBO J.* 14:2499-2508 (1995)]. This has also been suggested by COS cell studies with ZAP-70 carrying Tyr to Phe mutations at either Y492 or Y493 [Wange *et al.*, Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70, *J. Biol. Chem.* 270:18730-18733 (1995)].

These observations have led to a model describing the sequential activation of protein tyrosine kinases following TCR engagement [Weiss, A. and Littman, D. R., Signal transduction by lymphocyte antigen receptors, *Cell* 76:263-274 (1994); Cantrell, D., T Cell Antigen Receptor Signal Transduction Pathways, *Annu. Rev. Immunol.* 14: 259-274 (1996)]. Occupancy of the TCR causes the initial activation of Lck and/or Fyn leading to tyrosine phosphorylation of ITAMs. By virtue of its tandem SH2 domains, ZAP-70 then binds to these motifs [Wange *et al.*, Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor and CD3 from activated Jurkat T cells, *J. Biol.Chem.* 268:19797-19801 (1993); Iwashima *et al.*, Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases, *Science* 263:1136-1139 (1994)]. Recruitment of ZAP-70 to the ITAMs is required for activation of its kinase activity and for T cell activation, since agents that block recruitment prevent these events [Wange *et al.*, F2(Pmp)2-TAM 3, a novel competitive inhibitor of the binding of ZAP-70 to the T cell antigen receptor, blocks early T cell signaling, *J. Biol. Chem.* 270:944-948 (1995); Qian *et al.*, Dominant-negative Zeta-associated Protein 70 Inhibits T Cell Antigen Receptor Signaling, *J. Exp. Med.* 183:611-620 (1996)]. This sequence of events can be detected in cultured cell lines and in peripheral blood lymphocytes. However, in thymocytes and lymph node T cells, one can detect inactive, non-phosphorylated ZAP-70 bound to the basally tyrosine-phosphorylated TCR in the absence of activation [van Oers *et al.*, Constitutive tyrosine phosphorylation of the T-cell receptor (TCR) subunit: regulation of TCR-associated protein tyrosine kinase activity by TCR, *Mol. Cell. Biol.* 13:5771-5780 (1993); Wiest *et al.*, TCR activation of ZAP-70 is impaired in CD4+CD8+ thymocytes as a consequence of intrathymic interactions that diminish available p56lck, *Immunity* 4:495-504 (1996)]. The phosphorylation of ZAP-70 on Y493 by a Src-family

kinase results in the activation of ZAP-70 kinase activity [Chan *et al.*, Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function, *EMBO J.* 14:2499-2508 (1995); Wange *et al.*, Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70, *J. Biol. Chem.*270:18730-18733 (1995)]. Although this site shares sequence similarity with the so-called autophosphorylation sites defined on other PTKs, for ZAP-70 it is more accurate to refer to this as a transphosphorylation/activation site, since ZAP-70 is unable to phosphorylate this site, and phosphorylation of this site is required for full kinase activity. The mechanism of subsequent tyrosine phosphorylation events within the kinase domain at Y492, outside this region at Y292, and perhaps at other sites is not defined, but is likely to be due to autophosphorylation or transphosphorylation by other activated ZAP-70 molecules on adjacent ITAMs, although an additional role for a heterologous kinase has not been ruled out.

Clearly the role of phosphorylation of Y493 is to activate the kinase activity of ZAP-70; however, the roles played by phosphorylation of tyrosines 492 and 292 are less clear. In contrast to what is seen in Y493F mutants, the mutation of Y492 to Phe results in increased activity in ZAP-70 isolated from transfected COS cells and enhanced BCR signaling in $Syk^{-/-}$ B cells reconstituted with this ZAP-70 mutant [Wange *et al.*, Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70, *J. Biol. Chem.* 270:18730-18733 (1995); Guanghui *et al.*, Distinct tyrosine phosphorylation sites within ZAP-70 mediate activation and negative regulation of antigen receptor function, *Mol. Cell. Biol.*, In press (1996)]. Tyrosine Y492 may therefore serve a negative regulatory function in ZAP-70 when phosphorylated. Alternatively, mutation of Y492 to Phe may cause a loss of hydrogen bonding capacity that maintains the kinase in an inactive conformation when unphosphorylated [Wange *et al.*, Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70, *J. Biol. Chem.* 270:18730-18733 (1995)]. Mutation of Y292 to Phe, unlike the Y492F mutation, has no effect on the activity of purified ZAP-70, but, like the Y492F mutation, results in a hyperactivated state upon BCR stimulation in the $Syk^{-/-}$ chicken B cells [Guanghui *et al.*, Distinct tyrosine phosphorylation sites within ZAP-70 mediate activation and negative regulation of antigen receptor function, *Mol. Cell. Biol.*, In press (1996)]. This is consistent with phosphorylation of Y292 serving a

role as a binding site for regulatory molecules, such as SH2 domain-bearing protein tyrosine phosphatases (see below).

Recent reports have identified yet another class of PTKs, the Itk/Btk/Tec family, that may be involved in TCR signaling [reviewed in Desiderio, S. and Siliciano, J. D., The Itk/Btk/Tec Family of Protein-Tyrosine Kinases, *Chem. Immunol.* 59:191-208 (1994)]. This family of PTKs is characterized by having an N-terminal Plextrin Homology (PH) domain, and an SH2 and SH3 domain, in addition to the C-terminal kinase domain. This family of PTKs was first shown to be important in hematopoietic cell function when it was discovered that mutations in the Btk gene at the XLA locus cause X-linked agammaglobulinemia in man. Thus Btk, which is expressed predominantly in B lymphoid and myelomonocytic lineages, is required for normal B cell function. Itk is predominantly expressed in T cells. $Itk^{-/-}$ mice produce fewer thymocytes, and mature T cells isolated from these mice proliferate poorly in response to TCR stimulation, but respond normally to phorbol ester plus ionomycin [Liao, X. C. and Littman, D., Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. Immunity, 3:757-769 (1995)]. In Jurkat T cells, TCR cross-linking rapidly and transiently tyrosine-phosphorylates Itk, resulting in increased kinase activity in anti-Itk immunoprecipitates (Gibson *et al.*, The EMT/ITK/TSK (EMT) tyrosine kinase is activated during TCR signaling, *J. Immunol.* 156:2716-2722 (1996)]. The precise role of Itk in TCR signaling remains to be determined.

Characterization of pathways downstream of the PTKs depends on identification of their substrates. Detection of protein tyrosine phosphorylation by immunoblotting with specific anti-phosphotyrosine antibodies has revealed a number of kinase targets [Peri, K. G. and Veillette, A., Tyrosine protein kinases in T lymphocytes. Chem. Immunol. 59:19-39 (1994)]. In this fashion PLCγ1 was the first substrate, other than the TCR subunits, to be identified in T cells. Tyrosine phosphorylation of this enzyme is critical to its activation. Vav, a hematopoietic cell-specific proto-oncogene, and more recently, the proto-oncogene Cbl were also identified as being PTK substrates in T cells [Bustelo *et al.*, Product of the Vav protooncogene defines a new class of tyrosine protein kinase substrates, Nature 356: 68-74 (1992); Donovan *et al.*, The protein product of the c-cbl protooncogene is the 120-kDa tyrosine-phosphorylated protein in Jurkat cells activated via the T cell antigen receptor, *J. Biol. Chem.* 269:22921-22924 (1994)]. SH2-domain Leukocyte Protein (SLP-76), a prominent substrate, was recently isolated by virtue of an interaction with the Grb2 linker protein as described below [Jackman *et al.*, Molecular cloning of SLP-76, a 76-kDa tyrosine

phosphoprotein associated with Grb2 in T cells, *J. Biol. Chem.* 270:7029-7032 (1995)]. More difficult than identifying the proteins that become tyrosine phosphorylated upon TCR stimulation is determining which PTKs are directly responsible for a given phosphorylation event. As stated above it is clear that ITAM phosphorylation is a function of the Src-family kinases, as is phosphorylation of Y493 of ZAP-70. ZAP-70, at least *in vitro*, does not phosphorylate these sites.

Substrates of ZAP-70 have been more difficult to identify. Based on previous study of Syk, it has been shown that ZAP-70 can phosphorylate the cytoplasmic domain of erythrocyte band 3 (cdb3) and tubulin *in vitro* (Wange et al., 1995b; Isakov et al., 1996). Evidence that cdb3 and tubulin are genuine *in vivo* substrates of Syk has also been presented [Harrison *et al.*, Phosphorylation of human erythrocyte band 3 by endogenous p72syk, *J. Biol. Chem.* 269:955-959 (1994); Peters *et al.*, Syk, activated by cross-linking the B-cell antigen receptor, localizes to the cytosol where it interacts with and phosphorylates alpha-tubulin on tyrosine, *J. Biol. Chem.* 271:4755-4762 (1996)]. Whether tubulin is an *in vivo* substrate of ZAP-70 remains to be determined; however, it is interesting that tubulin is tyrosine phosphorylated in activated T cells, and both ZAP-70 and Vav have been found in association with tubulin [Ley *et al.*, Tyrosine phosphorylation of tubulin in human T lymphocytes, *Eur. J. Immunol.*, 24:99-106 (1994); Huby *et al.*, Interactions between the Protein-tyrosine Kinase ZAP-70, the Proto-oncoprotein Vav, and Tubulin in Jurkat T Cells, *J. Biol. Chem.*, 270:30241-30244 (1995)]. These findings further support the contention that cytoskeletal components may play an integral part in the early signal transduction steps. Of the molecules involved in TCR signal transduction that undergo tyrosine phosphorylation upon TCR engagement, only SLP-76 has been shown to be a substrate of ZAP-70 [Wardenburg *et al.*, Phosphorylation of SLP-76 by the ZAP-70 protein tyrosine kinase is required for T cell receptor function, *J. Biol. Chem.*, In press (1996)]. Tyrosine phosphorylation of SLP-76 mediates association with Vav (see below) (Wu *et al.*, Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation, *Immunity* 4:593-602 (1996)]. Of note is that sites likely to be phosphorylated in these three proteins, all share the sequence Asp-Tyr-Glu. Interestingly, the hematopoietic cell specific protein HS1, which has two Asp-Tyr-Glu sites, has been shown to be a substrate for ZAP-70 when HS1, ZAP-70 and Fyn are co-expressed in COS cells (Fusaki *et al.*, Physical and functional interactions of

protein tyrosine kinases, p59fyn and ZAP-70, in T cell signaling, *J. Immunol.* 156:1369-1377 (1996)]. The function of HS1 remains unknown.

C. Multimolecular Complexes in TCR signaling

While it is not intended that the present invention be limited to any particular mechanism of receptor-mediated signal transduction, it is believed that receptor activation leads to assembly of multimolecular complexes on the cytoplasmic domain of platelet-derived and epidermal growth factor receptors. These complexes comprise of multiple signaling molecules that often bind to the receptor by interaction at sites of tyrosine phosphorylation. Many of these signaling molecules are themselves modular, containing variable numbers of interaction domains such as (1) SH2, (2) PTB, (3) SH3 and (4) PH which bind, respectively to (1) pY (in the context of specific residues C-terminal of pY), (2) pY (in the context of specific residues N-terminal of pY), (3) proline-rich regions and (4) phosphotidylinositol (4,5) bisphosphate, inositol phosphates and certain proteins such as G protein βγ subunits. PH domains are thus thought to target some proteins to the plasma membrane. It is believed that, this modular architecture permits the assembly of large multimolecular complexes, as each signaling protein may bind to several different signaling proteins, which may themselves associate with a whole host of additional signaling molecules.

Proteins with the potential to become involved in multimolecular signaling complexes at the TCR include the receptor-proximal kinases themselves. The Src-family kinases contain an SH2, an SH3 and a unique N-terminal domain, all of which can be involved in protein-protein interactions [Bolen, J.B. . Nonreceptor tyrosine protein kinases. *Oncogene* 8: 2025-2031 (1993); Peri, K. G. and Veillette, A., Tyrosine protein kinases in T lymphocytes, *Chem.Immunol.* 59:19-39 (1994)]. In addition, lipid modification of consensus N-terminal sites stabilizes association of these PTKs with cellular membranes. The unique N-terminal domain of Lck binds to the co-receptor molecules CD4 or CD8, while the comparable region in Fyn binds to non-phosphorylated ITAMs. The SH2 and SH3 domains of Src-family PTKs form an intramolecular association with the tyrosine-phosphorylated C-terminal tail, inhibiting their kinase activity. The phosphorylation status of this negative regulatory tyrosine is controlled by the competing activities of the Csk PTK and the CD45 protein tyrosine phosphatase (PTP). In addition to activating the kinase activity, dephosphorylation of this residue by CD45 also increases the availability of the SH2 and SH3 domains for

interaction with other proteins (eg. Cbl, phosphatidylinositol 3-kinase [PI3-K] and ZAP-70) [Donovan *et al.*, The protein product of the c-cbl protooncogene is the 120-kDa tyrosine-phosphorylated protein in Jurkat cells activated via the T cell antigen receptor, *J. Biol. Chem.* 269:22921-22924 (1994); Vogel, L. B. and Fujita, D. J., The SH3 domain of p56lck is involved in binding to phosphatidylinositol 3'-kinase from T lymphocytes, *Mol. Cell. Biol.*, 13:7408-7417 (1993); Thome *et al.*, Syk and ZAP-70 mediate recruitment of p56lck/CD4 to the activated T cell receptor/CD3/ complex, *J. Exp. Med.*, 181:1997-2006 (1995)].

ZAP-70 is also capable of forming multimolecular complexes through its two SH2 domains and via tyrosine-phosphorylated SH2 domain acceptor sites. The only known function of the SH2 domains of ZAP-70 is to target the kinase to the two phosphorylated tyrosines of the ITAM [Wange *et al.*, Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor and CD3 from activated Jurkat T cells, *J. Biol. Chem.* 268:19797-19801 (1993); Iwashima *et al.*, Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases, *Science* 263:1136-1139 (1994)]. It is possible however that these SH2 domains have other targets when not engaged to the activated TCR, but this seems unlikely given the unique structure of the tandem SH2 domain of ZAP-70, which only permits high affinity binding to polypeptides possessing two phosphotyrosines within a prescribed distance, as is found in ITAMs [Hatada *et al.*, Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor, *Nature* 377:32-38 (1995)]. It is important to note that, unlike Syk, ZAP-70 is not activated by the binding of its tandem SH2 domains to ITAMs [Shiue *et al.*, Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based activation motifs of the high affinity receptor for IgE, *J. Biol. Chem.* 270:10498-10502 (1995); Neumeister *et al.*, Binding of ZAP-70 to phosphorylated T-cell receptor and enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins, *Mol. Cell. Biol.* 15:3171-3178 (1995); Isakov *et al.*, Purification and characterization of human ZAP-70 protein tyrosine kinase from a baculovirus expression system, *J. Biol. Chem.* 271:15753-15761 (1996)].

In addition to its role as an enzyme responsible for protein tyrosine phosphorylation, ZAP-70 appears also to function as an adaptor protein or scaffold upon which other signaling molecules assemble [Neumeister *et al.*, Binding of ZAP-70 to phosphorylated T-cell receptor and enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins, *Mol. Cell. Biol.* 15:3171-3178 (1995)]. Interestingly, Lck, Vav

and Cbl have all been found in association with ZAP-70 [Thome *et al.*, Syk and ZAP-70 mediate recruitment of p56lck/CD4 to the activated T cell receptor/CD3/ complex, *J. Exp. Med.* 181:1997-2006 (1995); Katzav *et al.*, The protein tyrosine kinase ZAP-70 can associate with the SH2 domain of proto-vav, *J. Biol. Chem.* 269:32579-32585 (1994); Fournel *et al.*, Association of tyrosine protein kinase ZAP-70 with the protooncogene product p120c-cbl in T lymphocytes, *J. Exp. Med.* 183:301-306 (1996)], and, at least for Lck and Vav, this association appears to involve the SH2 domains of these two proteins binding to phosphorylated tyrosines on ZAP-70. As mentioned previously tyrosines 69, 126, 178, 292, 492 and 493 have all been shown to be capable of accepting phosphate in an *in vitro* kinase reaction with Lck [Watts *et al.*, Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70, *J. Biol. Chem.* 269:29520-29529 (1994)]. It has been suggested that two other tyrosines (315 and 319) may be phosphorylatable, and phosphorylated peptides cognate for this region can disrupt the association of Vav with ZAP-70 (Katzav *et al.*, The protein tyrosine kinase ZAP-70 can associate with the SH2 domain of proto-vav, *J. Biol. Chem.* 269: 32579-32585 (994)] While the functional consequences of the ZAP-70 association with these signaling molecules remains to be determined, one can speculate that these associations ensure their efficient phosphorylation by ZAP-70 or associated PTKs, such as Lck. In addition, the association of Lck with tyrosine-phosphorylated ZAP-70 has been suggested to be required for recruitment of Lck-CD4 to the TCR [Thome *et al.*, Syk and ZAP-70 mediate recruitment of p56lck/CD4 to the activated T cell receptor/CD3/ complex, *J. Exp. Med.* 181:1997-2006 (1995)], thereby enhancing the assembly of a fully effective signaling complex.

The adaptor protein Grb2 is an example of a modular, so-called, linker protein, consisting entirely of a central SH2 domain flanked by two SH3 domains. In a number of growth factor receptor tyrosine kinase systems, Grb2 has been shown to couple these receptors to the Ras activator protein SOS [Downward, J.,The GRB2/Sem-5 adaptor protein, *FEBS Lett.* 338: 113-117 (1994)] As the Ras pathway was known to be required for lymphokine gene activation, a number of investigators sought to determine whether Grb2 was linked to this pathway in T cells. The Grb2-SOS interaction was indeed documented and SOS has been shown to be involved in Ras activation in T cells [Downward, J.,The GRB2/Sem-5 adaptor protein, *FEBS Lett.* 338: 113-117 (1994); Holsinger *et al.*, Signal

transduction in T lymphocytes using a conditional allele of Sos, *Proc. Natl. Acad. Sci. USA* 92:9810-9814 (1995)]. While, Shc, another linker protein, has been shown to link Grb2 to certain growth factor receptors, its role in TCR signaling remains controversial [Ravichandran *et al.*, Interaction of Shc with Grb2 regulates association of Grb2 with mSOS, *Mol. Cell. Biol.* 15:593-600 (1995); Osman *et al.*, A comparison of the interaction of Shc and the tyrosine kinase ZAP-70 with the T cell antigen receptor chain tyrosine-based activation motif, *J. Biol. Chem.* 270:13981-13986 (1995)].

In addition to the anticipated role of Grb2 in Ras activation, Grb2 was also found to bind several of the most prominent substrates of the TCR-associated PTKs [Motto *et al.*, In vivo association of Grb2 with pp116, a substrate of the T cell antigen receptor-activated protein tyrosine kinase, *J. Biol. Chem.* 269:21608-21613 (1994); Buday *et al.*, A complex of Grb2 adaptor protein, Sos exchange factor, and a 36-kDa membrane-bound tyrosine phosphoprotein is implicated in ras activation in T cells, *J. Biol. Chem.* 269:9019-9023 (1994); Reif *et al.*, SH3 domains of the adapter molecule Grb2 compete with two proteins in T cells: the guanine nucleotide exchange protein SOS and a 75-kDa protein that is a substrate for T cell antigen receptor-activated tyrosine kinases, *J. Biol. Chem.* 269:14081-14087 (1994)]. A 120kD protein that binds to Grb2 has been shown to be the proto-oncogene Cbl [Donovan *et al.*, The protein product of the c-cbl protooncogene is the 120-kDa tyrosine-phosphorylated protein in Jurkat cells activated via the T cell antigen receptor, *J. Biol. Chem.* 269:22921-22924 (1994); Fukazawa *et al.*, T cell activation-dependent association between the p85 subunit of the phosphatidyl 3-kinase and Grb2/phospholipase C-γ1-binding phosphotyrosyl protein, *J. BIol. Chem.* 270:36-38, 20177-20182 (1995); Meisner *et al.*, Interaction of Cbl with Grb2 and Phosphatidylinositol-3'-Kinase in Activated Jurkat Cells, *Mol. Cell. Biol.* 15:3571-3578 (1995)]. The Grb2-Cbl association is mediated via the N-terminal SH3 domain of Grb2, and is observed in resting and activated T cells. Affinity purification with a Grb2 fusion protein was used to isolate a 76kD protein, SLP-76, which binds to the C-terminal SH3 domain of Grb2 [Jackman *et al.*, Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells, *J. Biol. Chem.* 270, 7029-7032 (1995). The identity of pp36, a 36kD substrate, that binds to the SH2 domain of Grb2, has been identified to be "LAT', the composition claimed in the present invention. This protein plays a critical role in TCR signaling, as in addition to binding Grb2, it has also been found in association with PLCγ1 and PI3-K [Cantrell, D., T Cell Antigen

Receptor Signal Transduction Pathways, *Annu. Rev. Immunol.* 14: 259-274 (1996) and also see experimental section]. The tight membrane localization of pp36 provides a possible mechanism for recruitment of Grb2, PLCγ1 and PI3-K to the plasma membrane. Grb2-SOS activates the Ras-pathway when localized to the plasma membrane [Downward, J.,The GRB2/Sem-5 adaptor protein, *FEBS Lett.* 338: 113-117 (1994); Cantrell, D., T Cell Antigen Receptor Signal Transduction Pathways, *Annu. Rev. Immunol.* 14: 259-274 (1996)], while membrane localization of PLCγ1 and PI3-K permits phosphorylation of these proteins by membrane-associated PTKs, and provides access to lipid substrates.

The binding of SOS and Cbl to Grb2 is mutually exclusive, as proline-rich regions in these proteins compete for the same binding site [Meisner *et al.*, Interaction of Cbl with Grb2 and Phosphatidylinositol-3'-Kinase in Activated Jurkat Cells, *Mol. Cell. Biol.* 15:3571-3578 (1995)]. An interesting area of investigation is whether or not this competition plays a role in TCR signaling. It is also possible that Grb2 could mediate a complex between either pp36 and/or SLP-76 with Cbl or SOS. An additional consequence of formation of the Grb2-Cbl complex could depend on the ability of Cbl, itself, to serve as an adaptor protein. Recent studies demonstrate that Cbl can be found in complex with PI3-K, ZAP-70, 14.3.3τ and CrkL [Meisner *et al.*, Interaction of Cbl with Grb2 and Phosphatidylinositol-3'-Kinase in Activated Jurkat Cells, *Mol. Cell. Biol.* 15:3571-3578 (1995); Fukazawa *et al.*, T cell activation-dependent association between the p85 subunit of the phosphatidyl 3-kinase and Grb2/phospholipase C-γ1-binding phosphotyrosyl protein, *J. BIol. Chem.* 270:36-38, 20177-20182 (1995); Fournel *et al.*, Association of tyrosine protein kinase ZAP-70 with the protooncogene product p120c-cbl in T lymphocytes, *J. Exp. Med.* 183:301-306 (1996); Liu *et al.* Activation-modulated association of 14-3-3 proteins with Cbl in T cells. J. Biol. Chem. 271, 14591-14595 (1996); Reedquist *et al.*, Stimulation through the T cell receptor induces Cbl association with Crk proteins and guanine nucleotide exchange protein C3G, *J. Biol. Chem.* 271:8435-8442 (1996)]. CrkL is another adapter protein that in turn interacts with C3G, a nucleotide exchange factor for members of the Rac/Rho family of small G proteins.

Study of Vav and SLP-76 has led to the most significant recent insights into TCR signal transduction mechanisms. Vav, first identified as a transforming oncogene when truncated, consists of a number of interaction modules, 2 SH3 domains, one SH2 domain and one PH domain, along with regions sharing homology with Rac/Rho guanine nucleotide exchange proteins [Bustelo *et al.*, Product of the Vav protooncogene defines a new class of

tyrosine protein kinase substrates, *Nature* 356:68-74 (1992)]. Over-expression of this protein in Jurkat T cells results in enhanced basal activation of IL-2 promoters and further enhances the response to TCR signaling (Wu *et al.*, Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation, *Immunity* 4:593-602 (1995)]. Its proximal position in the TCR pathway was established by the observation that dominant negative Ras or Raf could block the Vav-mediated enhancement of TCR signaling. The position of Vav in the signaling cascade has been further delineated by the observation that the activity of Vav requires active PTKs and is not functional in a T cell line lacking Lck. Similar studies with SLP-76 over-expression also demonstrated an enhanced response to TCR engagement although without an increase in basal levels of activation (Motto *et al.*, Implications of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production, *J. Exp. Med.* 183:1937-1943 (1996)]. Over-expression of both Vav and SLP-76 causes a synergistic induction of basal and TCR-stimulated NFAT and IL-2 promoter activation [Wu *et al.*, Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation, *Immunity* 4:593-602 (1996)].

How these two signaling molecules ultimately affect transcriptional activity is unknown, but it has been shown that a specific interaction between Vav and SLP-76 is required for this activity [Wu *et al.*, Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation, *Immunity* 4:593-602 (1996)]. This association requires the SH2 domain of Vav and tyrosine phosphorylation of SLP-76. This association is required for the subsequent tyrosine phosphorylation of Vav, as crippling the SH2 domain of Vav prevents its tyrosine phosphorylation. Similarly, enhanced signaling does not occur if critical tyrosine residues (112, 128, 145) in SLP-76, presumably the binding sites for the Vav SH2 domain, are mutated to Phe. SLP-76, in turn binds two tyrosine-phosphorylated proteins of 130 (SLAP) and 62kD. An inactivating mutation within the SLP-76 SH2 domain prevents association of these proteins and significantly blocks the stimulatory effect of SLP-76 over-expression. Clearly Vav and SLP-76 have critical functional effects and the complex of these two proteins with others (eg. pp130 and pp62) is central to TCR function.

D. Tyrosine Phosphatases

The addition of phosphate to tyrosine residues catalyzed by tyrosine kinases induces many binding interactions as described. Regulation of these events by tyrosine phosphatases has equal significance both in initiating and quenching TCR signaling pathways [for review see McFarland *et al.*, Protein tyrosine phosphatases involved in lymphocyte signal transduction, *Chem. Immunol.* 59:40-61 (1994)]. Activation of Src family kinases by CD45 has already been mentioned. Recent evidence for interaction of the SHP-1 PTP with ZAP-70 suggests that regulation of this kinase or its substrates by SHP-1 may also be significant [Plas *et al.*, Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling, *Science* 272:1173-1176 (1996). This association is mediated by the SH2 domains of SHP-1, which bind to tyrosine phosphorylated ZAP-70 after TCR engagement. Engagement of the SH2 domains of SHP-1 by ZAP-70 stimulates the phosphatase activity with a consequent decrease in net ZAP-70 kinase activity. The functional significance of this interaction can be demonstrated by overexpression of wildtype SHP-1, which decreases IL-2 production in response to TCR stimulation, or conversely by overexpression of catalytically inactive SHP-1 (C453S), which has the opposite effect of enhancing IL-2 production. Other, as yet, unidentified PTPs are likely to have functional significance, and substrates of known PTPs remain to be defined. It is also interesting that SHP-1 has been found to associate with Vav in activated splenocytes and the EL4 T cell lymphoma [Kon-Kozlowski, M. et *al.*, The tyrosine phosphatase PTP1C associates with Vav, Grb2, and mSos1 in hematopoietic cells, *J. Biol. Chem.* 271:3856-3862 (1996)]. This association appears to involve the SH2 and flanking SH3 domains of Vav. As Vav and ZAP-70, and ZAP-70 and SHP-1 have also been shown to associate, determining the actual nature of the associations between these proteins will be required to more fully understand these signaling events. Another PTP that probably plays a role in down-regulating the TCR signal is SHP-2, which has been found to associate with the activation-upregulated T cell surface protein CTLA-4. Fyn, Lck and ZAP-70 are all hyperphosphorylated in CTLA-$4^{-/-}$ mice [Marengere *et al.*, Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4, *Science* 272:1170-1173 (1996)].

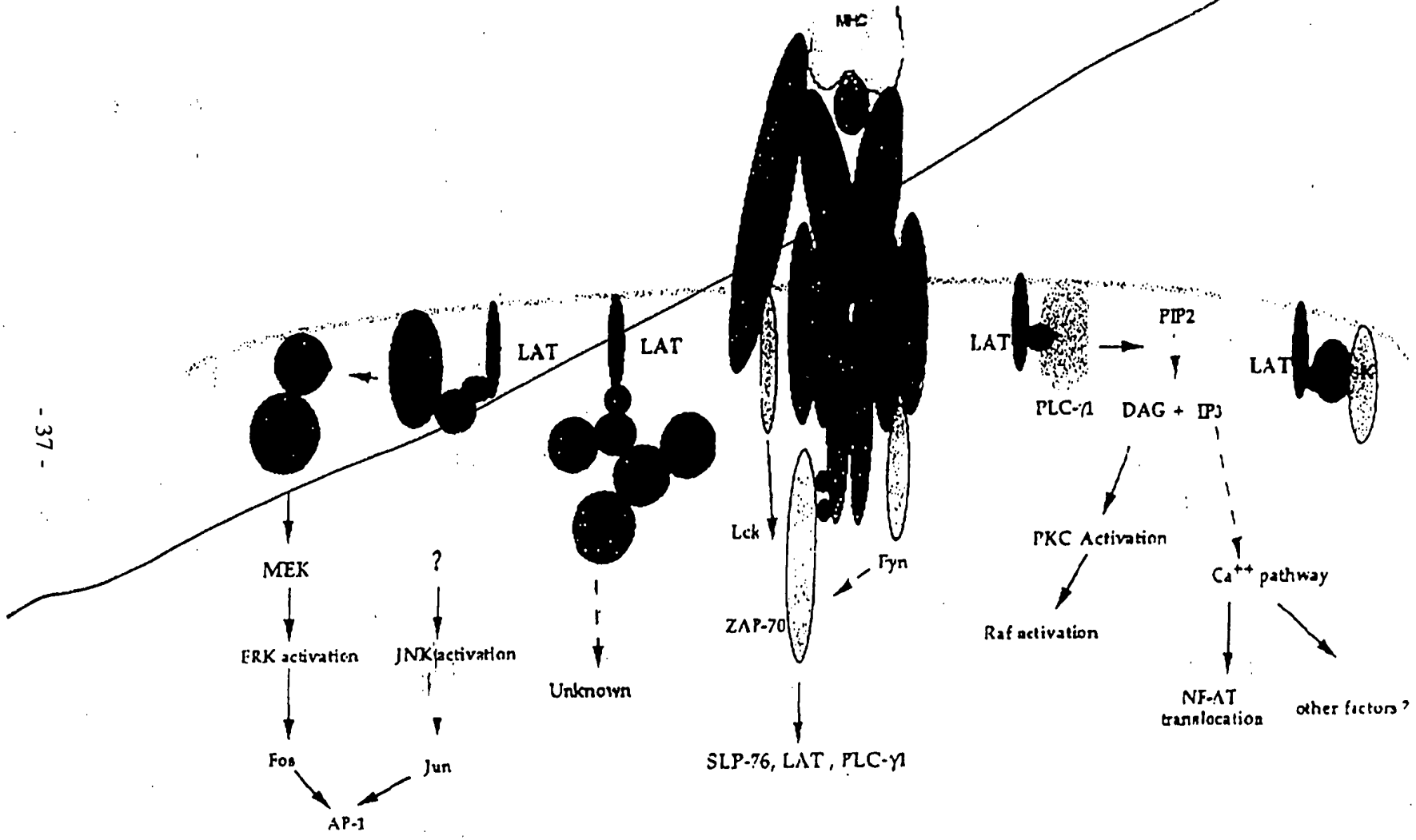
E. LAT as a ZAP-70 and/or Syk tyrosine kinase substrate

In the present invention, LAT, one of the most prominently tyrosine phosphorylated protein following TCR engagement has been identified. Deduced amino acid sequence identifies a novel integral membrane protein containing multiple potential tyrosine phosphorylation sites. This 36-38kD protein is capable of binding SH2 domains of Grb2, phospholipase C-γ1 and the p85 subunit of phosphoinositide 3-kinase and may play a central role as a molecule downstream of PTKs capable of binding critical PTK substrates and effector molecules. In the present invention, this protein is shown to be phosphorylated by ZAP-70 and/or Syk protein tyrosine kinases leading to recruitment of multiple signaling molecules. Its function is demonstrated by inhibition of T cell activation following overexpression of a mutant form lacking critical tyrosine residues (For details see experimental section), providing evidence that LAT plays an important role in linking the TCR to cellular activation. Therefore, the molecule has been named: LAT for linker for activation of T cells (See Schematic A).

F. Uses Of The Invention

The invention provides compositions and methods for identifying and testing tyrosine kinase ZAP-70 and/or Syk substrates and ZAP-70 and/or Syk kinase signaling pathway agonists and antagonists, and in particular, compositions comprising LAT (and fragments

LAT is a central molecule that links the TCR to cellular activation



Schematic A

thereof) which upon activation/tyrosine phosphorylation binds to ZAP-70 and/or Syk kinase and also to Grb2, PLC-γ-1, the p85 subunit of PI-3 kinase, and Cbl, Vav, SLP-76 and/or other critical signaling molecules, either directly or indirectly upon T cell activation.

The polypeptide LAT and fragments thereof, may have one or more LAT-specific binding affinities for particular ligands such as Grb2, PLC-γ-1, the p85 subunit of PI-3 kinase, and other critical signaling molecules, including the ability to specifically bind at least one natural human intracellular LAT-specific binding target or a LAT-specific binding agent such as a LAT-specific antibody or a LAT-specific binding agent identified in assays such as described below. Accordingly, the specificity of a LAT fragment for specific binding agents is confirmed by ensuring non-crossreactivity with other ZAP-70 and/or Syk substrates. Furthermore, preferred LAT fragments are capable of eliciting an antibody capable of distinguishing LAT from other LAT homologues.

1. Antibody Generation

Both polyclonal and monoclonal antibodies are obtainable by immunization with LAT, LAT fragments, mutant LAT, mutant LAT fragments, and LAT/LAT binding ligand complexes and either type is utilizable for immunoassays (as well as therapy). Polyclonal sera are readily prepared by injection of a suitable laboratory animal with an effective amount of the purified peptide, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Antibodies produced by this method are utilizable in virtually any type of immunoassay (see below).

The use of monoclonal antibodies directed to LAT, LAT fragments, mutant LAT, mutant LAT fragments, and LAT/LAT binding ligand complexes is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman, Basic Facts about Hybridomas, in Compendium of Immunology Vol II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature* 256: 495-499, 1975; *European Journal of Immunology* 6: 511-519, 1976).

Unlike preparation of polyclonal sera, the choice of animal is dependent on the availability of appropriate immortal lines capable of fusing with lymphocytes. Mouse and rat have been the animals of choice in hybridoma technology and are preferably used. Humans

can also be utilized as sources for sensitized lymphocytes if appropriate immortalized human (or nonhuman) cell lines are available. For the purpose of the present invention, the animal of choice may be injected with an antigenic amount, for example, from about 0.1 mg to about 20 mg of the peptide or antigenic parts thereof. Usually the injecting material is emulsified in Freund's complete adjuvant. Boosting injections may also be required. The detection of antibody production can be carried out by testing the antisera with appropriately labelled antigen. Lymphocytes can be obtained by removing the spleen of lymph nodes of sensitized animals in a sterile fashion and carrying out fusion. Alternatively, lymphocytes can be stimulated or immunized *in vitro*, as described, for example, in Reading, *Journal of Immunological Methods* 53: 261-291, 1982.

A number of cell lines suitable for fusion have been developed and the choice of any particular line for hybridization protocols is directed by any one of a number of criteria such as speed, uniformity of growth characteristics, deficiency of its metabolism for a component of the growth medium, and potential for good fusion frequency.

Intraspecies hybrids, particularly between like strains, work better than interspecies fusions. Several cell lines are available, including mutants selected for the loss of ability to secrete myeloma immunoglobulin.

Cell fusion can be induced either by virus, such as Epstein-Barr or Sendai virus, or polyethylene glycol. Polyethylene glycol (PEG) is the most efficacious agent for the fusion of mammalian somatic cells. PEG itself may be toxic for cells and various concentrations should be tested for effects on viability before attempting fusion. The molecular weight range of PEG may be varied from 1000 to 6000. It gives best results when diluted to from about 20% to about 70% (w/w) in saline or serum-free medium. Exposure to PEG at 37°C for about 30 seconds is preferred in the present case, utilizing murine cells. Extremes of temperature (i.e., about 45°C) are avoided, and preincubation of each component of the fusion system at 37°C prior to fusion can be useful. The ratio between lymphocytes and malignant cells is optimized to avoid cell fusion among spleen cells and a range of from about 1:1 to about 1:10 is commonly used.

The successfully fused cells can be separated from the myeloma line by any technique known by the art. The most common and preferred method is to choose a malignant line which is Hypoxthanine Guanine Phosphoribosyl Transferase (HGPRT) deficient, which will not grow in an aminopterin-containing medium used to allow only growth of hybrids and which is generally composed of hypoxthanine $1x10^{-4}$M, aminopterin $1x10^{-5}$M, and thymidine

$3x10^{-5}$M, commonly known as the HAT medium. The fusion mixture can be grown in the HAT-containing culture medium immediately after the fusion 24 hours later. The feeding schedules usually entail maintenance in HAT medium for two weeks and then feeding with either regular culture medium or hypoxthanine, thymidine-containing medium.

The growing colonies are then tested for the presence of antibodies that recognize the antigenic preparation. Detection of hybridoma antibodies can be performed using an assay where the antigen is bound to a solid support and allowed to react to hybridoma supernatants containing putative antibodies. The presence of antibodies may be detected by "sandwich" techniques using a variety of indicators. Most of the common methods are sufficiently sensitive for use in the range of antibody concentrations secreted during hybrid growth.

Cloning of hybrids can be carried out after 21-23 days of cell growth in selected medium. Cloning can be performed by cell limiting dilution in fluid phase or by directly selecting single cells growing in semi-solid agarose. For limiting dilution, cell suspensions are diluted serially to yield a statistical probability of having only one cell per well. For the agarose technique, hybrids are seeded in a semi-solid upper layer, over a lower layer containing feeder cells. The colonies from the upper layer may be picked up and eventually transferred to wells.

Antibody-secreting hybrids can be grown in various tissue culture flasks, yielding supernatants with variable concentrations of antibodies. In order to obtain higher concentrations, hybrids may be transferred into animals to obtain inflammatory ascites. Antibody-containing ascites can be harvested 8-12 days after intraperitoneal injection. The ascites contain a higher concentration of antibodies but include both monoclonals and immunoglobulins from the inflammatory ascites. Antibody purification may then be achieved by, for example, affinity chromatography.

Recombinant antibodies are also contemplated, and in particular, single chain antibodies prepared according to Pastan *et al.*, U.S. Patent No. 5,608,039 (hereby incorporated by reference). In particular, humanized antibodies are contemplated. Such antibodies are non-human antibodies in which some or all of the amino acid residues are replaced with the corresponding amino acid residue found in a similar human antibody. Humanization thereby reduces the antigenic potential of the antibody.

It is not intended that the present invention be limited to specific portions of LAT for the generation of antibodies. However, the cytosolic tail of LAT is a preferred portion for

generating specific antibodies. Such antibodies can be subjected to differential absorption using GST-LAT truncations to enhance specificity for different parts of the molecules.

In another embodiment, antibodies to phosphorylated LAT are contemplated. In such embodiments, phosphorylated expressed LAT protein or synthetically prepared phosphotyrosine containing peptides with the LAT sequence are used. Such antibodies are contemplated as useful for studying (and detecting) the phosphorylation of LAT and are contemplated as antibodies useful for binding activated LAT. Such reagents can be introduced into cells to target specific LAT interactions.

2. Antibody Assays

The presence of the LAT polypeptide may be accomplished in a number of ways. A wide range of immunoassay techniques are available as can be seen by reference to U.S. Patent Nos. 4,016,043 and 4,424,279 and 4,018,653 (herein incorporated by reference). This, of course, includes both single-site and two-site, or "sandwich", assays of the non-competitive types, as well as in the traditional competitive binding assays.

Sandwich assays are among the most useful and commonly used assays. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen secondary complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of a tertiary complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent.

In the typical forward sandwich assay, a first antibody having specificity for LAT or antigenic parts thereof, is either covalently or passively bound to a solid surface. The solid

surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated at 25°C (or higher) for a period of time sufficient to allow binding. The incubation period will vary but will generally be in the range of about 1 minute to 2 hours, and more typically 2-40 minutes. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody.

An alternative method involves immobilizing LAT (or a sample containing LAT) and then exposing the immobilized LAT to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of LAT and the strength of the reporter molecule signal, LAT may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the first antibody/LAT complex to form a tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores, luminescent molecules or radionuclide containing molecules (i.e. radioisotopes).

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for peroxidase conjugates, 1,2-phenylenediamine, 5-aminosalicyclic acid, or toluidine are commonly used. It is also

possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the tertiary complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope.

Immunofluorescent and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed. It will be readily apparent to the skilled technician how to vary the procedure to suit the required purpose.

3. LAT Binding Ligands

The present invention contemplates using LAT, LAT fragments, mutant LAT, and mutant LAT fragments to identify LAT Binding Ligands. Exemplary natural intracellular binding targets include protein tyrosine kinases which comprise one or more LAT binding sites and phosphotyrosine peptide fragments thereof, and protein kinases such as activated ZAP-70 and/or Syk tyrosine kinases and fragments of such targets which are capable of LAT-specific binding. Other natural LAT binding targets and fragments thereof are readily identified by screening cells, membranes and cellular extracts and fractions with the disclosed materials and methods and by other methods known in the art. For example, two-hybrid screening using ZAP-70 and/or Syk kinases are used to identify intracellular targets which specifically bind such LAT fragments. Preferred LAT fragments retain the ability to specifically bind at least one ZAP-70 and/or Syk kinase binding sites.

A wide variety of molecular and biochemical methods are available for generating and expressing LAT fragments and ZAP-70 and/or Syk kinase. For example, the ZAP-70 and/or Syk kinase and LAT or fragments thereof may be obtained by chemical synthesis, expression in bacteria such as *E. coli* and eukaryotes such as yeast or vaccinia or baculovirus-based expression systems, insect cells etc., depending on the size, nature and quantity of the LAT or fragment. The LAT fragments are of length sufficient to provide a novel peptide. As used herein, such peptides are at least 5, usually at least about 6, more usually at least about 8, most usually at least about 10 amino acids. LAT fragments may be present in a free state or bound to other components such as blocking groups to chemically insulate reactive groups (e.g. amines, carboxyls, etc.) of the peptide, fusion peptides or polypeptides (i.e. the peptide may be present as a portion of a larger polypeptide), etc. The same applies to the binding ZAP-70 and/or Syk kinase fragments, other LAT binding ligands etc.

The LAT fragments maintain binding affinity of not less than six, preferably not less than four, more preferably not less than two orders of magnitude less than the binding equilibrium constant of a full-length native LAT to the binding target under similar conditions.

Particular LAT fragments or deletion mutants are shown to function in a dominant-negative fashion. Overexpression of particular LAT fragments, for e.g. mLAT are also shown to block NFAT and AP-1 transcriptional activation mediated by OKT3 stimulated Jurkat T cells. Such LAT fragments provide therapeutic agents, e.g. when delivered by intracellular immunization--transfection of susceptible cells with nucleic acids encoding such mutants. The claimed LAT and LAT fragments are isolated, partially pure or pure and are typically recombinantly produced. As used herein, an "isolated" peptide is unaccompanied by at least some of the material with which it is associated in its natural state and constitutes at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by weight of the total protein (including peptide) in a given sample; a partially pure peptide constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of the total protein in a given sample; and a pure peptide constitutes at least about 70%, preferably at least about 90%, and more preferably at least about 95% by weight of the total protein in a given sample.

The invention provides LAT-specific binding agents, methods of identifying and testing such agents, and their use in diagnosis, therapy and pharmaceutical development. For

example, LAT-specific agents are useful in a variety of diagnostic applications, especially where disease or disease prognosis is associated with immune dysfunction resulting from improper expression of LAT or due to the signaling pathways being modified by LAT. In addition, LAT specific agents are useful in detecting clinical conditions of LAT deficiency, LAT mutations by assessing the levels of LAT expression and/or LAT phosphorylation in clinical samples, by developing assays such as PCR , Immunoblotting etc. Novel LAT-specific binding agents include LAT-specific antibodies; novel nucleic acids with sequence similarity to that of LAT; isolated ZAP-70 and/or Syk kinase binding domains; Grb2 binding domains; other natural intracellular binding agents identified with assays such as one- and two-hybrid screens; non-natural intracellular binding agents identified in screens of chemical libraries, etc.

The invention also provides nucleic acids encoding the subject LAT and LAT fragments thereof, the said nucleic acids may be part of LAT-expression vectors and may be incorporated into recombinant cells for expression and screening, transgenic animals for functional studies, etc. In addition, the invention provides nucleic acids sharing substantial sequence similarity with that of one or more wild-type (WT) LAT nucleic acids. Substantially identical or homologous nucleic acid sequences hybridize to their respective complements under high stringency conditions, for example, at 55°C and hybridization buffer, comprising 50% formamide in 0.9M saline/0.09M sodium citrate (SSC) buffer and remain bound when subject to washing at 55°C with the SSC/formamide buffer. Where the sequences diverge, the differences are preferably silent, i.e. or a nucleotide change providing a redundant codon, or conservative, i.e. a nucleotide change providing a conservative amino acid substitution.

The nucleic acids find a wide variety of applications including use as hybridization probes, PCR primers, therapeutic nucleic acids, etc. for use in detecting the presence of LAT genes and gene transcripts, for detecting or amplifying nucleic acids with substantial sequence similarity such as LAT homologs and structural analogs, and for gene therapy applications (including antisense approaches). Given the subject probes, materials and methods for probing cDNA and genetic libraries and recovering homologs are known in the art. Preferred libraries are derived from human immune cells, especially cDNA libraries from differentiated and activated human lymphoid cells. In one application, the subject nucleic acids find use as hybridization probes for identifying LAT cDNA homologs with substantial

sequence similarity. These homologs in turn provide additional LAT and LAT fragments for use in binding assays and therapy as described herein.

Therapeutic LAT nucleic acids are used to modulate, usually reduce, cellular expression or intracellular concentration or availability of active LAT. These nucleic acids are typically antisense: single-stranded sequences comprising complements of the disclosed LAT nucleic acids. Antisense modulation of LAT expression may employ LAT antisense nucleic acids operably linked to gene regulatory sequences. Cells are transfected with a vector comprising an LAT sequence with a promoter sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to endogenous LAT encoding mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for stable extrachromosomal maintenance or integration. Alternatively, single-stranded antisense nucleic acids that bind to genomic DNA or mRNA encoding a LAT or human LAT (hLAT) fragment may be administered to the target cell, in or temporarily isolated from a host, at a concentration that results in a substantial reduction in hLAT expression. For gene therapy involving the transfusion of hLAT transfected cells, administration will depend on a number of variables that are ascertained empirically. For example, the number of cells will vary depending on the stability of the transfused cells. Transfusion media is typically a buffered saline solution or other pharmacologically acceptable solution. Similarly the amount of other administered compositions, e.g. transfected nucleic acid, protein, etc., will depend on the manner of administration, purpose of the therapy, and the like.

The subject nucleic acids are often recombinant, meaning they comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome. An isolated nucleic acid constitutes at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by weight of total nucleic acid present in a given fraction. A partially pure nucleic acid constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given fraction.

The invention provides efficient methods of identifying pharmacological agents or drugs which are active at the level of LAT modulatable cellular function, particularly LAT mediated T cell antigen receptor signal transduction. Generally, these screening methods

involve assaying for compounds which interfere with hLAT activity such as hLAT-ZAP-70 and/or LAT-Syk kinase binding, LAT-Grb2 binding, etc. The methods are amenable to automated, cost-effective high throughput drug screening and have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs. Target therapeutic indications are limited only in that the target cellular function (e.g. gene expression) be subject to modulation, usually inhibition, by disruption of the formation of a complex comprising a hLAT or hLAT fragment and one or more natural hLAT intracellular binding targets. Since a wide variety of genes are subject to ZAP-70 and/or Syk kinase regulated signaling, target indications may include viral, bacterial and fungal infections, metabolic disease, genetic disease, cell growth and regulatory disfunction, such as neoplasia, inflammation, hypersensitivity, etc. Frequently, the target indication is related to either immune dysfunction or selective immune suppression.

A wide variety of assays for binding agents are provided including labelled *in vitro* protein-protein and immunoassays for protein binding or complex formation, cell based assays such as two or three hybrid screens, transient transfection and co-immunoprecipitation assays, etc. For example, three-hybrid screens are used to rapidly examine the effect of transfected nucleic acids, which may, for example, encode combinatorial peptide libraries or antisense molecules, on the intracellular binding of hLAT or hLAT fragments to intracellular hLAT targets. Convenient reagents for such assays (e.g. GST fusion partners) are available in the art.

hLAT or hLAT fragments used in the methods are usually added in an isolated, partially pure or pure form and are typically recombinantly produced. The hLAT or fragment may be part of a fusion product with another peptide or polypeptide, e.g. a polypeptide that is capable of providing or enhancing protein-protein binding, sequence-specific nucleic acid binding or stability under assay conditions (e.g. a tag for detection or anchoring). The assay mixtures comprise at least a portion of a natural intracellular hLAT binding target such as ZAP-70 and/or Syk kinase subunit domain or Grb2 binding domains. The assay mixture also comprises a candidate pharmacological agent. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the limits of assay detection. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds. Small organic compounds have a molecular weight of more than 50 yet less than

about 2,500, preferably less than about 1000, more preferably, less than about 500. Candidate agents comprise functional chemical groups necessary for structural interactions with proteins and/or DNA, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups, more preferably at least three. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the aforementioned functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof, and the like. Where the agent is or is encoded by a transfected nucleic acid, said nucleic acid is typically DNA or RNA. Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. In addition, known pharmacological agents may be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs. A variety of other reagents may also be included in the mixture. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used. The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the LAT specifically binds the cellular binding target, portion or analog. The mixture components can be added in any order that provides for the requisite bindings. Incubations may be performed at any temperature which facilitates optimal binding, typically between 4 and 40°C, more commonly between 15 and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening, and are typically between 0.1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours. After incubation, the presence or absence of specific binding between the hLAT and one or more binding targets is detected by any convenient way. For cell-free binding type assays, a separation step is often used to separate bound from unbound

components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate which may be any solid from which the unbound components may be conveniently separated. The solid substrate may be made of a wide variety of materials and in a wide variety of shapes, e.g. microtiter plate, microbead, dipstick, resin particle, etc. The substrate is chosen to maximize signal to noise ratios, primarily to minimize background binding, for ease of washing and cost. Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting reservoir such as a microtiter plate well, rinsing a bead (e.g. beads with iron cores may be readily isolated and washed using magnets), particle, chromatographic column or filter with a wash solution or solvent. Typically, the separation step will include an extended rinse or wash or a plurality of rinses or washes. For example, where the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific binding such as salts, buffer, detergent, nonspecific protein, etc. may exploit a polypeptide specific binding reagent such as an antibody or receptor specific to a ligand of the polypeptide. Detection may be effected in any convenient way. For cell based assays such as one, two, and three hybrid screens, the transcript resulting from hLAT-target binding usually encodes a directly or indirectly detectable product (e.g. galactosidase activity, luciferase activity, etc.). For cell-free binding assays, one of the components usually comprises or is coupled to a label. A wide variety of labels may be employed, essentially any label that provides for detection of bound protein. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. The label may be appended to the protein e.g. a phosphate group comprising a radioactive isotope of phosphorous, or incorporated into the protein structure, e.g. a methionine residue comprising a radioactive isotope of sulfur. A variety of methods may be used to detect the label depending on the nature of the label and other assay components. For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected. Labels may be directly detected through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc. For example, in the case of radioactive labels, emissions may be detected directly, e.g. with particle counters or indirectly, e.g. with scintillation cocktails and counters. The methods are particularly suited to automated high throughput drug screening.

Candidate agents shown to inhibit hLAT-target binding provide valuable reagents to the pharmaceutical industries for animal and human trials. For example, LAT fragments capable of blocking T cell activation, find use in treating disease associated with undesirable cell growth, differentiation, particularly immune cell differentiation, and hypersensitivity/allergy. In addition, they find use in the inhibition of autoreactive T cells (autoimmunity), inhibition of production of deleterious T cell products (such as cytokines, lymphokines) and/or T cell activities (such as cytotoxicity, etc). Also, the invention finds use in treating diseases associated with cell movement, particularly as changes in motility are important in growth and differentiation. Thus, it could be relevant in the treatment of cancer, immunological diseases, autoimmune diseases, graft rejection and others. For therapeutic uses, the compositions and agents disclosed herein may be administered by any convenient way, preferably parenterally, conveniently in a physiologically acceptable carrier, e.g., phosphate buffered saline, saline, deionized water, or the like. Typically, the compositions are added to a retained physiological fluid such as blood or synovial fluid. Generally, the amount administered will be empirically determined, typically in the range of about 10 to 1000μg/kg of the recipient. For peptide agents, the concentration will generally be in the range of about 100 to 500 μg/ml in the dose administered. Other additives may be included, such as stabilizers, bactericides, etc. These additives will be present in conventional amounts.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see, generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., and Current Protocols in Molecular Biology (1996) John Wiley and Sons, Inc., N.Y.) which is incorporated herein by reference) which are provided throughout this document. All the information contained therein is incorporated herein by reference.

Oligonucleotides can be synthesized on an Applied BioSystems oligonucleotide synthesizer [for details see Sinha *et al.*, Nucleic Acids Res. 12:4539 (1984)], according to specifications provided by the manufacturer. Complementary oligonucleotides are annealed by heating them to 90°C in a solution of 10 mM Tris-HCl buffer (pH 8.0) containing NaCl (200 mM) and then allowing them to cool slowly to room temperature. For binding and turnover assays, duplex DNA is purified from native polyacrylamide (15% w/v) gels. The band corresponding to double-stranded DNA is excised and soaked overnight in 0.30 M sodium acetate buffer (pH 5.0) containing EDTA (1 mM). After soaking, the supernatant is extracted with phenol/chloroform (1/1 v/v) and precipitated with ethanol. DNA substrates are radiolabeled on their 5'-OH group by treatment with [g-^{32}P]ATP and T4 polynucleotide kinase. Salts and unincorporated nucleotides are removed by chromatography on Sephadex G columns.

Assays for detecting the ability of agents to inhibit or enhance LAT binding provide for facile high-throughput screening of agent banks (e.g., compound libraries, peptide libraries, and the like) to identify antagonists or agonists. Such ZAP-70 and/or syk tyrosine kinase signaling pathway antagonists and agonists may be further developed as potential therapeutics and diagnostic or prognostic tools for diverse types of infectious diseases, autoimmune diseases and hereditary diseases, and cancer.

A. Drug Screening Assays for Agonists and Antagonists

In some embodiments, LAT (and in particular, fragments of LAT) are useful in drug screening assays designed to identify drugs that interfere with the specific binding of ZAP-70 and/or Syk kinases with its substrate as well as ZAP-70 and/or Syk kinase activity, and thereby block the activation of downstream signaling molecules.

In preferred embodiments, the invention provides an isolated LAT polypeptide, or a fragment thereof, having Grb2-specific binding affinity. The invention provides nucleic acids encoding the LAT polypeptide and LAT fragments as part of expression vectors for introduction into cells. The invention provides methods of identifying intracellular molecules which interact with LAT or LAT fragments, as well as exogenous agents (*i.e.* drugs) which disrupt or enhance the binding of LAT and/or fragments thereof to such intracellular targets.

The claimed polypeptide LAT and LAT fragments find particular use in screening assays for agents or lead compounds for agents useful in the diagnosis, prognosis or treatment of disease, particularly disease associated with undesirable cell growth, cell

movement, differentiation and cell proliferation. One such assay involves forming mixtures of 1) LAT (or fragments thereof) and 2) an intracellular LAT-binding ligand, in the presence or absence of 3) a prospective drug candidate. The mixtures are made under conditions that permit the binding of the intracellular LAT-binding ligand to LAT (or fragments thereof) and the mixtures are then analyzed for the presence of such binding. A difference in such binding in the presence of such a drug candidate indicates that the agent is capable of modulating the binding of LAT (or fragments thereof) to an intracellular LAT-binding ligand. The assays of the present invention provide for facile high-throughput screening of compounds suspected to be able to inhibit such binding (e.g., compound libraries, peptide libraries, and the like) to identify potential drug candidates.

The compositions and methods of the present invention can be used to identify and screen for drugs that block the following LAT interactions (also see detailed description of specific embodiments):

1) Development of drugs that prevent ZAP-70 and/or other kinases from interacting and phosphorylating LAT

2) Development of drugs that prevent LAT from interacting with ZAP-70 or from getting phosphorylated.

3) Development of drugs that prevent binding of phosphorylated LAT to ligands such as Grb2 etc.

4) Development of drugs that prevent binding of Grb2 (or other ligands) to phosphorylated LAT.

An example of a drug screening assay, that can be employed to test for potential drug candidates that inhibit the specific binding of Grb2 with LAT, is given below. The suspect drug candidates are tested whether they block the binding of Grb2 to the full length LAT, or preferably to Grb2 fused to glutathione-S-transferase (GST). GST-Grb2 or GST-Grb2 is immobilized on glutathione-agarose beads and incubated for 1 hour with the indicated amount or different doses of the drug candidate. In parallel assays, the mutant LAT or αLAT antibodies are used as controls. Lysates from Jurkat T cells treated for 2min with or without C305 are incubated with the immobilized GST-Grb2 in the presence or absence of the drug candidate (or in parallel assays with the mutant LAT). Bound proteins are eluted with sodium dodecyl sulfate-containing buffer, separated by SDS-PAGE and immunoblotted with αLAT. GST-Grb2 binds to LAT in C305 stimulated Jurkats, but not unstimulated cells. Alternatively, one can screen for drugs that block Grb2 binding to GST-LAT. Treatment

with mutant LAT (mLAT) or αLAT antibodies inhibits the binding of GST-Grb2 to LAT in a dose-dependent fashion.

In other embodiments, drug screening assays that can be employed for testing potential drug candidates (for agonists), are those that overcome the inhibition of transcriptional activation of NF-AT (involved in TCR-mediated transcriptional events). Details of these assays are given below. Jurkat TAg cells, can be stably transfected with mutant LAT(Y171/191F), (in parallel assays with the wild-type or vector only), together with a reporter construct for secreted alkaline phosphatase (SEAP) driven by the NF-AT response elements. The suspect drug candidates are tested whether they overcome the inhibition of the transcriptional activation of NF-AT in OKT3 stimulated mLAT transfected Jurkat cells. (For more details, see methodology and example 7 in the experimental section). Identification of agonists are particularly useful in treatment of clinical conditions of LAT deficiency.

B. Structural and Functional Investigation of LAT.

In other embodiments, signaling molecules that bind to LAT can be identified. Based on structural and functional features of LAT (See below and experimental Section), LAT may serve as an adapter molecule that recruits multiple signaling molecules to Ligand-LAT complexes. It is believed that one or more of these proteins may link these complexes to cellular functions. Signaling proteins are predicted either to bind to pTyr within LAT in response to ligand or to bind constitutively to LAT. Identification of LAT binding partners also would provide insight into T cell, NK cell and mast cell signaling pathways. The techniques for studying the interaction of the protein(s) of interest with LAT and analyzing their role in cellular functions are as described below and in the experimental section.

i. Structure-function analysis of LAT

In some embodiments, LAT can be subjected to mutagenesis followed by analysis of function, i.e., to understand how does LAT regulate the following and how do mutations of LAT affect the following :

A. Functional assays: -all can be performed in T cells (Jurkat, normal T cells from humans or mice), mast cells, NK cells. Alternatively, non-lymphocyte cells can be studied by transient or stable expression of LAT or LAT mutants. Additionally one can express necessary kinases, receptors, accessory molecule and relevant indicator molecules. Studies can be performed *in vitro*, in intact cells or in animal models.

i). ***Protein kinase assays***

a. protein tyrosine kinases-Lck, Fyn, ZAP-70, Syk, Itk

b. protein serine kinases-Raf, MEK (all), ERK (all), PAK, MEKK (all), JNKK (all), JNK (all) and all related identified and, as of now, unidentified members of these protein serine/threonine kinase families.Protein kinase C (all family members). One can identify novel kinases regulated directly or indirectly by LAT.

ii). ***Calcium assays***-one can analyze elevations from extra-and intra-cellular sites

iii). ***Assays of small G proteins***- (including, but not limited to Ras, Rac, Rho, cdc42, Rap) and regulators of small G protein function (including, but not limited to SOS, GAP, Vav)

iv). ***Assays of protein binding*** -including, but not limited to those molecules shown to be directly or indirectly bound to LAT (Grb2, Grap, phospholipase C, PI-3 kinase, Vav, SLP-76, Cbl)

v). ***Assays of transcriptional regulation***- including, but not limited to NFAT, AP-1, Fos, Jun

vi). ***Localization assays using indirect immunofluorescence and GFP chimeras.*** Assay by light, confocal, imaging microscopy

vii). ***Assays of T cell cellular function***- including, but not limited to cell shape, cell size, cell motility, cell migration, cell adhesion

viii). ***Assays of tyrosine and serine/threonine phosphorylation of LAT***- By using mass spectrometry, chromatography and antibodies specific for all regions of LAT

ix). ***Generate panels of anti-LAT antibodies***- antibodies that bind phosphorylated and non-phosphorylated regions of LAT.

B. Mutagenesis of LAT

In preferred embodiments, Structure-function analysis of LAT can be performed by mutagenesis of: i) Tyrosine residues, singly and in combinations; ii) Truncations from carboxy end and truncations of amino terminal end of the cytosolic domain (the cytosolic domain is defined approximately by amino acids 28 to 233) followed by re-generation of in-frame sequence; point mutations as indicated following truncations; iii) Deletion of putative transmembrane domain (the transmembrane domain is defined approximately by residues 5 to 27) and mutagenesis of residues in putative transmembrane domain; iv) Mutations of charged residues and cysteine residues near putative transmembrane domain.

ii. Analysis of LAT interactions

In other embodiments, analysis of LAT interactions with other signaling proteins can be performed as given below.

A. One can study the interactions of LAT with the signaling molecules as described in the above section and in the experimental section. Also, one can map sites of interactions as stated above (mutagenesis, region-specific antibodies, etc.).

B. One can determine new and additional interactions with additional intracellular molecules, by Protein purification, such as Affinity purification using LATprotein, mRNA or cDNA as a probe. By employing two and three hybrid analysis to identify interacting molecules.

C. One can determine interactions with integral membrane proteins including, but not limited to any component of the T cell, B cell, mast cell or NK cell antigen or immunoglobulin receptors, including, but not limited to accessory or co-receptor molecules such as CD4, CD8, CD28.

iii. Analysis of the role of LAT in T cell, mast cell and NK cell function

In some embodiments, the function of LAT can be ascertained in various cells as given below.

A. In T cells, one can study T cell activation, T cell apoptosis, T cell anergy, T cell development and differentiation, T cell helper and killer function, T cell lymphocyte/cytokine production. In addition, one can generate transgenic animals overexpressing wild-type or mutant LAT; generation of LAT knock-outs. Also, one can generation LAT deficient T cell line as recipient for mutant forms of LAT.

B. In Mast cells, one can study granule release, cytokine production, second messenger production, gene transcription

C. In NK cells one can study various functions, such as cytotoxity, cytokine and second messenger production, gene transcription

iv. Structural analysis of LAT

In other embodiments, structural analysis of LAT can be performed as given below.

A. Mutation of LAT to generate cytosolic fragment followed by analysis of structure by NMR and/or crystallography.

B. Comparison of structure of mutant forms of LAT. Comparison of structure of non-phosphorylated and phosphorylated forms of LAT.

C. Modeling small molecule interactions with structural models of LAT.

iv. Inhibitors of LAT function

In preferred embodiments, inhibitors of LAT function can be identified and/or generated as given below.

A. Generation of antibodies that bind LAT and block function, including monoclonal, single chain antibodies, recombinant antibodies

B. Identify small molecules that bind LAT, by screening libraries of organics for binders, peptide binders, molecular design

C. Generation of anti-sense molecules to decrease LAT expression

D. Assay as above for function, interaction, localization, etc.

vi. LAT-mediated immunodeficiency

In preferred embodiments, the compositions and methods of the present invention can be employed for developing diagnostic assays

A. Screen various patients with immunodeficiencies to find patients lacking LAT or with mutated LAT. Define clinical condition(s) characterized by LAT deficiency

B. Establish cell lines from such patients. Reconstitute with type. Define mutations in LAT; reexpress mutant form (patient form) in LAT deficient cell line. Use naturally occurring mutants for structure-function analysis.

C. Develop screening test for LAT deficiency or mutation.

D. Develop genetic therapy for LAT deficiency; bone marrow or T cell reconstitution.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. In the experimental disclosure which follows, the following methodology apply:

Antibodies, Immunoprecipitation and Western Blotting: Antibodies used in the experiments were rabbit polyclonal anti-Grb2, anti-Grb2 cross-linked to agarose, anti-Cbl (C-15), and anti-Sam68 from Santa Cruz Biotechnology. Monoclonal anti-PLC-γ1, anti-

phosphotyrosine (4G10) and anti-Vav, rabbit anti-p85 polyclonal were from Upstate Biotechnology. Others were anti-Flag M2 (Kodak), and anti-phosphotyrosine antibody (PY20, Transduction Laboratory). PY20 was chemically cross-linked to protein A-sepharose using dimethyl pimelimidate (DMP). GST fusion proteins were crosslinked to glutathione-sepharose with 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide (EDC, Pierce). Anti-LAT antibodies were generated against a GST fusion protein containing the cytosolic portion of LAT (aa 31-233).The following antibodies were used: rabbit polyclonal anti-LAT antiserum (Zhang, *et al.*, LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation, *Cell* 92:83-92, 1998), anti-ZAP-70 (Wange, *et al.*, Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70, *J. Biol. Chem.* 270:18730-18733, 1995), anti-Lck (Samelson *et al.*, Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor, *Proc. Natl. Acad. Sci. USA* 87:4358-4362, 1990), anti-CD3ε (OKT3, Kung *et al.*, Monoclonal antibodies defining distinctive human T cell surface anigens, *Science* 206:347-349, 1979), monoclonal anti-myc (9E10) and monoclonal anti-PLCγ1 (a gift from Dr. S. G. Rhee, NHLBI, NIH).

Jurkat T cells (ATCC) were removed from culture, washed and resuspended at 10^8 cells/ml in RPMI. Cells were either stimulated with OKT3 ascites (1:100) (ATCC) or C305 (1:50) for 2 minutes or left untreated and lysed in 1% Brij lysis buffer (Wange *et al.*, Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. *J. Biol. Chem. 270*, 18730-18733, 1995).

For treatment with calf intestine alkaline phosphatase (CIP), immunoprecipitates from 10^7 cells were incubated with 10 units of CIP (Boehringer) in 40 µl CIP reaction buffer for 1 hour.

Purification of LAT: 10^{11} Jurkat E6.1 cells were stimulated for 1.5 min at 10^8/ml in RPMI-1640 with OKT3 ascites (1:100) at 37 °C. Immediately after stimulation, cells were washed with cold RPMI quickly and membrane fractions were prepared by Dounce homogenization and centrifugation. Membrane fractions were then extracted with 1% Brij lysis buffer, and insoluble material was removed by centrifugation. Membrane protein extracts were heated for 10 min at 75°C, followed by centrifugation to remove insoluble material. This supernatant was loaded onto a PY20 column, which was washed sequentially with lysis buffer containing 1% Brij 97 (25mM Tris-HCl ph 7.6, 150mM NaCl, 5mM EDTA, 1mM sodium orthoranadate, 10 µg/ml each of leupeptin, aprotinin, p-nitrophenyl-p-

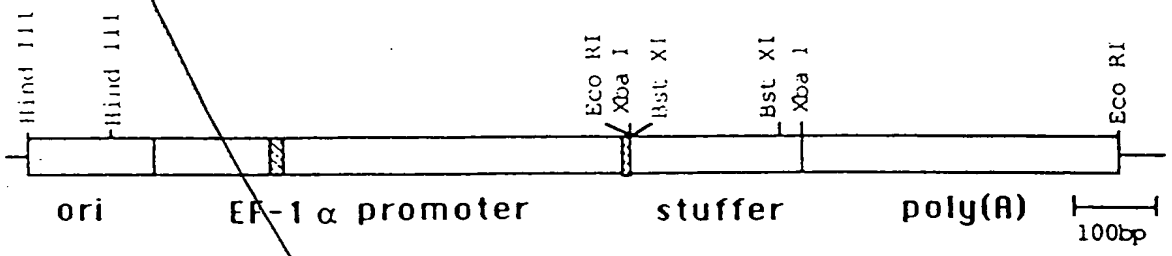
guanidinobenzoate), 1% CHAPS (3-[(3-cholamidopropyl)-dimethylamminio]-1-propanesulfonate), and 0.1% CHAPS. Phospho-tyrosine containing proteins were eluted with 0.1 % CHAPS buffer containing 10mM phenyl phosphate. Eluted proteins were concentrated and subjected to SDS-PAGE. The band for p36-38 was excised and subjected to *in gel* S-carboxyamidomethylation followed by tryptic digestion as described, without the addition of 0.02% Tween. The resulting peptide mixture was separated by microbore HPLC using a Zorbax C18 1.0mm by 150 mm reverse-phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Optimum fractions were chosen based on differential UV absorbance at 205nm, 277nm and 292nm, peak symmetry and resolution; then further screened for length and homogeneity by matrix-assisted laser desorption time-of-flight spectrometry (MALDI-MS) on a Finnigan Lasermat 2000 (Hemel England). Tryptic peptides were submitted to automated Edman degradation on an Applied Biosystems Procise 494 or 477A protein sequencer (Foster City, CA).

cDNA cloning and Northern blotting: A YT lambda ZAP cDNA library (10^6 plaques) was screened with a random primed probe made from the *Eco* RI/*Pst* I fragment of an EST clone (Genbank #w74254). 20 overlapping phage clones were isolated. The cDNAs were excised from lambda phages with the rapid excision kit from Stratagene. For Northern analysis, blots with poly(A)$^+$ RNA from different human tissues (ClonTech) were probed with a radiolabeled fragment of LAT cDNA or human β-actin cDNA under high stringency conditions according to the manufacturer's instruction.

Construction of LAT in expression vector and mutagenesis: The myc tag was fused to the C-terminus of LAT by replacing the *Pst* I/*Hind* III fragment of LAT in pBluescript (SK-) (Stratagene) with a double stranded oligonucleotide fragment encoding the myc tag sequence (SMEQKLISEEDLN). Flag-tagged LAT was constructed by replacing the *Pst* I/*Cla* I fragment of LAT in pBluescript with a double stranded oligonucleotide fragment encoding the FLAG sequence (DYKDDDDK). The tagged LAT cDNAs were cloned into the pcDNA3 (InVitrogen) or pEF/BOS vector (See Schematic B, and Mizushima and Nagata *et al.*, *Nuc. Acid Res.*, 18: 5322, 1990).

As shown in Schematic B, pEF-BOS carries the SV40 replication origin (311 bp of *Eco*RII G fragment), the promoter region of human EF-1α chromosomal gene (1.2 kb), the stuffer fragment (450 bp) from CDM8 vector (See Seed, B. *Nature* 329:840-842, 1987), and poly(A) adenylation signal from human EF-1α chromosomal gene (1.2 kb), the stuffer

fragment (450 bp) from CDM8 vector and poly(A) adenylation signal from human G-CSF cDNA (700 bp *Eco*811~ *Eco*RI DNA fragment) (Nagata S *et al., Nature* 319: 415-418, 1986) avin *Hind*III-*Eco*RI site of pUC119. The promoter region of EF-1α gene is from nucleotide position 373 to 1561 (Uetsuki *et al., J. Biol. Chem.*, 264:5791-5798, 1989), which includes 203 bp 5' flanking region, 33 bp first exon, 943 bp first intron and 10bp of the part of the second exon located at 20 bp upstream of the ATG codon. The size of the pEF-BOS is 5.8 kb, and the cDNA to be expressed can be inserted at *Bst*XI site using *Bst*XI adapter, or *Xba*I site using *Xba*I linker. Mutagenesis of Y171 to F and Y191 to F was done by PCR.



The structure of pEF-BOS. The boxes indicate SV40 origin, human EF-1α promoter region, stuffer from CDM8 and poly(A) adenylation site, respectively. The slashed areas in the EF-1α promoter region are first exon and the part of second exon, respectively. The lines flanking boxes are the sequence of pUC119. Major recognition sites for restriction enzymes are shown.

Schematic B

Stable and transient transfections: Transfection of 293T cells was by the calcium phosphate method. Cells were harvested 36 hours after transfection. For transient transfection of Jurkat/Tag cells, $2x10^7$ cells in 0.4 ml RPMI-1640, 25 mM Hepes, 2 mM Glutamine were incubated with pSX-NFAT/SEAP or pSX-AP-1/SEAP with 20μg of pEF/LAT(wt), pEF/LAT(Y171/191F) or pEF/BOS vector DNA as control. 24 hours after transfection, transfected cells were stimulated with OKT3 ascites (1:1000) or PMA (10 ng/ml) plus ionomycin (Sigma) (1.5μM). SEAP assay was done as described (Spencer *et al.*, Controlling signal transduction with synthetic ligands. *Science 262*, 1019-1024, 1993; Berger *et al.*, Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells, *Gene* 66:1-10, 1988). For stable transfection of Jurkat E6.1, 10^7 cells in 0.4 ml RPMI were electroporated using the same conditions as above. Stable transfectants were selected in the presence of 1.5 mg/ml G418 (Gibco/BRL).

***Immunofluorescence Staining and Confocal Microscopy*:** HeLa cells transfected with the cDNA for LAT were grown on sterile glass coverslips overnight prior to antibody staining. Jurkat T cells were stained in suspension, and the cells were mounted onto coverslips immediately prior to analysis. Immunofluorescence staining were performed as described (Sloan-Lancaster *et al.*, Regulation of ZAP-70 intracellular localization: visualization with the green fluorescent protein. *J. Exp. Med.* 186, 1713-1724. 1997) with anti-LAT at 1:500 dilution for T cells or 1:1000 dilution for HeLa cells (ATCC) , or monoclonal anti-Lck (Santa Cruz Biotech, CA) at 1:500 dilution.

***Ligands of LAT*:** The nucleic acid and amino acid sequences for ZAP-70 (GENBANK ACCESSION No. L05148), Syk Kinase (GENBANK ACCESSION No. Z29630), Grb2 (GENBANK ACCESSION No. M96995), Vav (GENBANK ACCESSION No.X16316) and Cbl (GENBANK ACCESSION No.X57110) are available.

Purification of GEM Fractions and Subcellular Fractionation: $5X10^7$ cells were lysed on ice in 1 ml 1% Triton in MNE buffer (25 mM MES pH 6.5, 150 mM NaCl, 5 mM EDTA), dounced 10 times, and mixed with 1 ml 80% sucrose made with MNE buffer. After transfer of the lysate to the centrifuge tube, 2 ml 30% sucrose in MNE buffer was overlaid, then 1 ml 5% sucrose in MNE was overlaid. After centrifugation for 16-18 hours at 200,000g in a Beckman SW55Ti, 0.4 ml gradient fractions were collected from the top of the gradient. For purification of GEMs from OKT3 stimulated cells, cells were spun down quickly after stimulation and then lysed in 1 ml 1% Triton in 25 mM Tris-Cl pH 7.6, 150

mM NaCl, 5 mM EDTA, 30 mM pyrophosphate, 10 mM glycerol phosphate and 1 mM sodium orthovanadate. Lysates were prepared for sucrose gradient ultracentrifugation as above.

Mutagenesis and Subcloning: Cysteine to alanine mutations (position 26 and 29) of LAT cloned into the pCEFL expression vector (a gift from Dr. S. Gutkind, NIDR, NIH) were made with the Stratagene Quickchange kit. The LAT transmembrane domain deletion mutant (residues 1-22) was made by cloning a double-stranded linker annealed with two oligonucleotides (AATTCGCCGCCATGGCACTGTGTG and TGCACACACAGTGCCATGGCGGCG), *Apa* L1/*Xba* I fragment from pEF/LAT-myc into pCEFL *Eco* RI/*Xba* I sites.

Transfection, Labeling of Jurkat Cells and 293T Cells, and Immunofluorescence: Transient and stable transfection of Jurkat cells were performed as described (Zhang, *et al.*, LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation, *Cell* 92:83-92, 1998). For labeling with [^{3}H] palmitate, 2X10^7 Jurkat cells were removed from culture and resuspended in 1 ml RPMI 1640 containing 5% dialyzed fetal calf serum (FCS), 5 mM sodium pyruvate and 0.5 mCi [^{3}H]-palmitate for 3 hours. 293T cells were transfected with LAT constructs using the calcium phosphate method in 6 well plates. 24 hours after transfection, 293T cells were labeled with 0.5 mCi [^{3}H]-palmitate in 1 ml DMEM containing 5% dialyzed FCS and 5 mM sodium pyruvate, for 3 hours. The gels were fixed, treated with Enlightning (Dupont) for 30 min, dried, and exposed to film for 3-4 weeks. Immunofluorescence staining and confocal microscopy were done with 293T cells transfected with LAT constructs as described (Zhang, *et al.*, LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation, *Cell* 92:83-92, 1998).

***Normal Tissues and Cell Populations*:** Paraffin embedded normal lymphoid tissues included lymph nodes showing various forms of reactive changes (10 cases), thymuses obtained during cardiac surgery or surrounding thymomas (3), spleens removed after trauma or because of immune thrombocytopenia (4), bone marrows (6), and small intestine (2). In addition, hematopoietic tissues from three embryos aged 11-12 weeks of gestation were analyzed. Freshly frozen samples of reactive lymph nodes (3), spleen (1), and thymus (1) were also used.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood after Ficoll-Hypaque gradient centrifugation and depleted of plastic adherent cells. For purification of polyclonal natural killer (NK) or T cell populations, PBMC were incubated

with anti-CD3 monoclonal antibody (JT3A, gift by Dr. A. Moretta, University of Genova, Italy) for 30 minutes at 4°C, followed by treatment with goat-anti mouse-coated dynabeads (Dynal, Oslo, Norway) for 30 minutes at 4°C. The resulting CD3 negative lymphocyte populations, containing approximately 1% CD3+ cells, 20-30% HLADR+ cells and 70-80% CD16+CD56+ cells, were cultured in rIL-2 (Cetus Corp. Emeryville, CA). In order to obtain polyclonally activated T cell-enriched lymphocyte populations, PBMC were stimulated with 0.1% (vol/vol) PHA (Gipco Ltd, Paisley, Scotland) for 24 hours and then cultured in rIL-2.

Neoplastic Tissues: Two-hundred and sixty-four cases of nodal and extranodal hematolymphoid neoplasms were gathered from different institutions; all neoplasms had been previously characterized immunophenotypically on paraffin sections, and in many cases on frozen sections as well. All lymphomas were classified according to the International Lymphoma Study Group Classification, and included all major subtypes of Hodgkin's and Non-Hodgkin's lymphomas.

Fixatives and Tissue and Cell Processing: Tissue samples had been fixed in various fixatives, including buffered formalin, B5, Bouin, and Hollande, and embedded in paraffin. Bone marrow biopsies were fixed in B5 for 3 hours and decalcified in 0.1 M EDTA disodium salt aqueous solution for 2-8 hours. Fresh tissues were immediately frozen after biopsy in liquid nitrogen and stored at -800C until used.

CD3+CD16-CD56- T cell and CD3-CD16+CD56+ NK cell populations were washed three times in 0.9% NaCl solution, resuspended at a concentration of $5x10^6$ cells/ml and utilized for cytospin preparations (100 μl/each slide). Slides were air dried for 24 hours, then fixed in -20°C absolute ethanol for 30 minutes, dried and used for immunocytochemical staining.

Immunostaining of Fixed Sections: Details on the production and characterization of the rabbit anti-LAT antibody are reported elsewhere (Zhang, *et al.*, LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation, *Cell* 92:83-92, 1998). The immunostaining for LAT was performed on paraffin sections after antigen retrieval in microwave (3 boiling cycles, 5 minutes each at 750 watts power, with an interval of 1 minute between cycles) in citrate buffer, pH 6.0; the polyclonal antibody anti-LAT was applied at a dilution of 1:800 in TRIS-HCl buffer, pH 7.2-7.4, for 45 minutes, and was followed by biotinylated anti-rabbit antibody (30 minutes) and peroxidase-conjugated streptavidin-biotin complex (30 minutes) (Bio-S.P.A., Milan, Italy). On cryostat sections, the procedure was similar, but the microwave heating was avoided. Sections were air dried for

18 hours, and LAT was applied at the dilution of 1:100. On cytospins, the sample was subjected to microwave heating once.

In all cases of T-cell lymphomas and anaplastic large cell lymphomas (ALCL), serial paraffin sections were also stained with a polyclonal antibody anti-CD3 (Dako, Milan) (1:200; microwave antigen retrieval in 1 mM EDTA buffer, pH 8.0, 2x5' cycles). Furthermore, the cases of ALCL were also evaluated for their reactivity with the monoclonal antibody ALK1 (Dako) (1:10; microwave antigen retrieval in citrate buffer, 3x5' cycles), which recognizes a formalin-resistant epitope in the nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) chimeric protein. Both CD3 and ALK1 immunostaining were performed using the same indirect immunoperoxidase technique adopted for LAT. Thymuses from embryos were also stained with polyclonal antibodies anti-CD3 and anti-TdT (Dako) (1:200; microwave antigen retrieval in 1 mM EDTA buffer, pH 8.0, 3x5' cycles, overnight incubation of the primary antibody).

Analysis of the distribution of LAT on PMBC was performed using two-colour fluorescence cytofluorometric analysis (FACS) (Ortho Cytoron Absolute) as previously described [135]. For FACS analysis of LAT in combination with monoclonal antibodies JT3A (IgG2a, anti-CD3), KD1 (IgG2a, anti-CD16) and C218 (IgG1, anti-CD56) (all antibodies provided by Dr. A. Moretta), membranes were permeabilized with 0.2% saponin (Sigma) in phosphate buffered saline (PBS) (pH 7.6) for 5 minutes, after fixation with 4% paraformaldehyde in PBS. As second reagents, fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit antibody (Dako) and phycoerythrin (PE)-conjugated isotype specific goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL) were used.

EXAMPLE 1

In this example, the purification process of LAT is described. Stimulation of the TCR on the Jurkat human T cell line by crosslinking with either C305 (anti-TCRβ, See Fig.1A) or OKT3 (anti-CD3ε, not shown) monoclonal antibodies induced the tyrosine phosphorylation of multiple proteins, most prominently, p36-38. To define the role of p36-38 in T cell signaling, the protein was purified from activated Jurkat cells. Since, p36-38 appears to be membrane-associated, membrane fractions were prepared from OKT3 stimulated Jurkat cells and extracted with Brij97 detergent. The extracted membrane proteins were then heat-treated, which induced precipitation of about 2/3 of the protein, but not of p36-38. Anti-phosphotyrosine antibodies were used for affinity purification of

phosphotyrosine-containing proteins. These were then specifically eluted with phenyl phosphate, concentrated, and resolved on SDS-PAGE. The p36-38 band was excised, digested *in gel* with trypsin, and the resultant tryptic peptides were resolved by microbore reverse-phase HPLC (See Fig. 1B).

EXAMPLE 2

In this example, peptide sequencing and cDNA cloning of the LAT protein is described. Peptides from five HPLC fractions were either microsequenced or subjected to mass spectrometry. The peptide from fraction 40 had a molecular weight of 1721.9 daltons (See Figure 1C). The residues at position 1, 2, 3, 11 and 15 could not be assigned unambiguously. The available sequence was then used to search the Genbank data base with the BLAST algorithm, and an EST clone from human fetal heart was found to encode the peptide fragment. Review of the Edman sequence data at the ambiguous positions was consistent with the EST sequence. The predicted molecular weight of the putative tryptic fragment predicted from the EST sequence was 1641.8 daltons. The 80.1 daltons difference suggested that a phosphate moiety of this molecular weight could be present at the Tyr residue. Two peptides from peaks 82 and 31 were shown by sequence analysis to be derived from the known tyrosine kinase substrate SLP-76. It was not clear whether these fragments of this 76kD substrate were cleaved physiologically or during protein purification. To avoid sequencing other peptides derived from SLP-76, the masses of subsequent peptides were determined. With this strategy, it was concluded that the peptide in fraction 55 was likely to originate from SLP-76, but the masses of two peptides in fraction 48 could not be of SLP-76 origin. The sequence of the larger of these two peptides was not found in the EST clone or Genbank. Interestingly, fraction 48 contained a small amount of a second peptide with a molecular weight of 1641.6, 80.3 daltons less than the mass of the peptide isolated and sequenced from peak 40. A partial amino acid sequence revealed that this peptide was the non-phosphorylated form of the peak 40 peptide.

A cDNA library from YT cells was probed with the *Eco* RI/ *Pst* I fragment of the EST clone. Thirty positive clones were isolated, of which the longest was 1.6 kb. Determination of the nucleotide sequences of these clones predicted a protein that contains the two tryptic peptide sequences found in the isolated peptides (underlined in Fig.1D). Through comparison of the EST and cDNA sequences, it was found that the first 380 nucleotides in the EST clone were not present in the cDNA. This unknown sequence might

be derived from intronic sequence. Probes from the human cDNA were used to screen a murine adult thymus library. The murine amino acid sequence obtained was 66% identical to the human sequence (Fig.7D).

The coding sequence of human p36-38 began at nucleotide 58. The first methionine, not found within a consensus Kozak sequence, was followed by an open reading frame predicting a 233 amino acid polypeptide with a calculated molecular mass of 24,985 Daltons. One cDNA clone had an 87 nucleotide insertion at position 396, encoding an additional 29 amino acids. Of the 233 amino acids found in the majority of clones, there were a total of 39 negatively charged residues (16 Asp and 23 Glu), but only 11 positively charged residues (2 Lys and 9 Arg). The high relative negative charge could have resulted in retarded migration on SDS-PAGE leading to an apparent molecular weight of 36-38kD, in excess of the predicted mass. The deduced amino acid sequence contained no domain homologous to known tyrosine, serine/threonine, or lipid kinases, nor did it contain SH2, SH3, or PTB domains. A single region of hydrophobic amino acids extended from residues 5 to 27, and may form an α-helical transmembrane domain. Two of the four residues N-terminal to this region were negatively charged in both human (two Glu) and murine (one Glu and one Asp). Positively charged residues (one Arg in the human and two Arg in the murine sequence), were found immediately after this hydrophobic domain. The relative charge difference across this putative transmembrane domain may determine the orientation of the molecule in the membrane, resulting in an extremely short extracellular amino-terminus and a long cytosolic tail.

Consistent with the prominent tyrosine phosphorylation of the protein in activated cells, the predicted cytosolic domain of human p36-38 contained ten tyrosines, of which nine were conserved between the human and murine proteins. Based on studies of Songyang and colleagues (Songyang *et al.*, Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk and Vav. *Mol. Cell. Biol. 14*, 2777-2785, 1994), after phosphorylation there were five potential binding sites for Grb2 SH2 domains (YxN) at Tyrosines 110, 127, 171, 191 and 226. Tyr171 and Tyr191 have an identical Grb2-binding motif (YVNV). A binding motif for the SH2 domain of the p85 subunit of PI3 kinase was not observed. A sequence related to the consensus binding motif for N-terminal and C-terminal PLC-γ1 SH2 domains (YLVV, Tyr132) were found to be present. Since the p36-38 protein most likely functioned as a docking protein capable of recruiting signaling

molecules, and in view of the observed features of its sequence, the name LAT for linker for activation of T cells was given.

To confirm that the cDNA clone encodes the full length LAT protein, and to resolve the discrepancy between the predicted and apparent molecular weight, the human LAT cDNA was subjected to *in vitro* transcription and translation. The reaction product contained a 38 kDa band that was not present when control DNA was used, confirming that the complete LAT coding sequence was present (not shown). The LAT cDNA was modified to include an epitopic tag (myc) at the C-terminus, cloned into the pcDNA3 expression vector and transfected into 293T cells (See Figure 2A). Immunoprecipitation and blotting demonstrated a 40kD protein in the transfected, but not non-transfected cells. These results also indicated that the LAT cDNA clone contained the entire coding region of the protein.

To demonstrate the LAT protein in T cells, rabbit polyclonal antibodies were raised against the cytosolic portion of LAT fused to GST. The resulting antiserum was used to detect LAT in lysates of activated Jurkat cells (See Figure 2B). The LAT antiserum precipitated and detected a doublet of 36 and 38kD, which comigrated with bands detected with antiphosphotyrosine antibodies. Repetitive immunoprecipitation with this antiserum depleted most of p36-38 proteins from stimulated Jurkat lysate (data not shown). Phosphorylation of LAT partially interfered with the detection of this protein as demonstrated by enhanced blotting following dephosphorylation of the immunoprecipitate with calf intestinal phosphatase (CIP). This antiserum enabled the determination that LAT was rapidly tyrosine phosphorylated upon stimulation with OKT3 (See Figure 2C). Maximal phosphorylation of LAT was seen after stimulation for 15 seconds, and after 2 min LAT was rapidly dephosphorylated. These phosphorylation kinetics were also seen in whole cell lysates (not shown). The results in this example demonstrate that the LAT cDNA encodes a protein that migrates on SDS-PAGE with an apparent molecular weight of 36-38kD, and which is tyrosine phosphorylated, following TCR activation.

EXAMPLE 3

In this example, tissue and cellular distribution of the LAT protein is described. Northern blot analysis was performed with poly $(A)^+$ RNAs isolated from different adult human tissues. Two transcripts (2.0 and 1.6 Kb) were seen predominantly in thymus, peripheral blood, and at a low level of expression in spleen. There were no transcripts of LAT found in other tissues (See Fig. 3A and data not shown). LAT expression was assayed

in human and rat cell lines of hematopoietic origin. LAT mRNA was only found in Jurkat, YT (NK-like cells) and RBL (a rat mast cell line) (See Figure 3B). From these data, it was concluded that LAT is expressed in T cells, NK cells, and mast cells, but not in B cells (Raji and Jiyoye) or monocytes (THP1).

The cellular localization of LAT was analyzed by immunofluorescence in COS cells, which were transiently transfected with LAT cDNA, fixed, permeabilized and incubated sequentially with anti-LAT and TRITC-conjugated goat anti-rabbit antibodies. Visualization with confocal microscopy showed that LAT was localized to the plasma membrane, and a juxtanuclear intracellular compartment (data not shown). No staining was detected in untransfected COS cells. Plasma membrane localization of endogenous LAT was also specifically detected in Jurkat cells. When cells were simultaneously stained with anti-LAT and anti-Lck antibodies, LAT and Lck were seen to co-localize at the plasma membrane and in an intracellular compartment.

EXAMPLE 4

This example shows that LAT is a substrate of ZAP-70 and Syk protein tyrosine kinases. Src family and Syk family kinases are known to be directly involved in TCR-mediated signaling. To examine which tyrosine kinases can phosphorylate LAT, FLAG-tagged LAT was transiently coexpressed with Lck, Fyn, ZAP-70 or Syk, alone or in certain combinations in 293T cells. Phosphorylation of LAT was detected by anti-phosphotyrosine antibody blotting. There was no tyrosine phosphorylation of LAT when it was expressed alone in 293T cells (See Fig. 4A). When LAT was coexpressed with Lck, constitutively activated Lck (Y505F) or Fyn, only coexpression of activated Lck resulted in minimal tyrosine phosphorylation of LAT. Co-transfection with Syk resulted in marked LAT tyrosine phosphorylation. However ZAP-70 alone failed to phosphorylate LAT. ZAP-70, unlike Syk, is known to require activation by a Src kinase family member. Consequently, when LAT was cotransfected with ZAP-70 and Lck or ZAP-70 and Fyn, LAT was tyrosine phosphorylated to a level comparable to that induced by Syk. At least two unidentified tyrosine phosphorylated proteins of 90 and 60kD co-precipitated with LAT under these conditions. Likewise, the association of LAT with endogenous Grb2 linker protein required LAT tyrosine phosphorylation induced by either Syk or the combination of ZAP-70 with either Lck or Fyn. The results in this example indicate that LAT is a substrate of ZAP-70

and Syk, and that tyrosines in LAT involved in binding to the Grb2 SH2 domain are phosphorylated by these PTKs.

EXAMPLE 5

In this example, association of LAT with Grb2, Grap, PI3K and PLC-γ1 is shown in 293T cells. To investigate the association of LAT with critical signaling molecules, FLAG-tagged LAT in the pcDNA3 vector was cotransfected in 293T cells with plasmids expressing HA-tagged Grb2, Myc-tagged Grap, HA-tagged p85 subunit of PI3K or PLC-γ1, in the presence or absence of both ZAP-70 and Lck. LAT was immunoprecipitated with anti-FLAG antibody, and associated proteins were detected by blotting with specific antibodies. As shown in Figure 4B, LAT tyrosine phosphorylation and association with endogenous Grb2 was induced by cotransfection with ZAP-70 and Lck as in Fig. 4A. When LAT and the two PTKs were coexpressed with HA-tagged Grb2, both endogenous Grb2 and HA-Grb2 bound to phosphorylated LAT (HA-Grb2 migrates more slowly than endogenous Grb2 on SDS-PAGE). Myc-tagged Grap, the Grb2-like protein, when coexpressed with LAT in the presence of the two PTKs, also associated with phosphorylated LAT. However, in this immunoprecipitation no endogenous Grb2 was bound to phosphorylated LAT, suggesting that Grap competes for Grb2 binding sites. HA-tagged p85 or PLC-γ1 also associated with phosphorylated LAT. Some endogenous Grb2 bound to LAT, but less than when LAT was cotransfected only with ZAP and Lck. Surprisingly, some association of LAT with PLC-γ1 was found, without co-transfection of the PTKs, suggesting either binding to undetectable tyrosine phosphorylation sites or binding via an SH2-independent mechanism.

The above experiments were designed to demonstrate LAT associations by isolation of LAT followed by detection of associated molecules. The reciprocal approach was also performed to confirm these associations. 293T cells were co-transfected with FLAG-tagged LAT, ZAP-70 and Lck, and either HA-tagged Grb2 or myc-tagged Grap (Fig.4C). As above, immunoprecipitation of LAT allowed detection of that fraction of Grb2 or Grap associated with LAT. In addition, immunoprecipitation of either Grb2-HA or Grap-myc allowed detection of the fraction of LAT bound to either adaptor protein. Similar experiments were performed in cells co-transfected with HA-p85 or PLC-γ1. Immunoprecipitation of these proteins revealed associated LAT (not shown). Additional experiments were performed to determine if the PTKs, ZAP-70 or Lck, could bind to LAT. No associations between LAT and either PTK were detected (not shown).

EXAMPLE 6

In this example, association of LAT with Grb2, PLC-γ1 and PI3K is shown in Jurkat T cells. The ability of LAT to associate with signaling proteins in T cells was next evaluated. The interaction of Grb2 with p36-38 is thought to be mediated by the Grb2 SH2 domain. To test this, lysates prepared from resting and activated Jurkat cells stimulated with C305 were incubated with the immobilized fusion proteins, GST-Grb2, GST-Grb2 with an SH2 domain mutation (R86K), and GST-Grb2 with mutations at both SH3 domains (P49L/G203R). Only GST-Grb2 and GST-Grb2 with SH3 mutations bound a 36-38 kDa tyrosine phosphorylated protein from C305 stimulated Jurkat lysate (Figure 5A). Mutation at the SH2 domain of Grb2 abolished the interaction between this 36-38 kDa protein and GST-Grb2. Subsequently the same membrane was probed with anti-LAT antibodies which confirmed that this p36-38 is LAT. LAT was not detected in material subjected to purification with GST-Grb2 from unstimulated cells or with GST-Grb2 containing the SH2 mutation.

To demonstrate the interactions in T cells directly, lysates from unstimulated and stimulated Jurkat T cells were subjected to immunoprecipitation with anti-Grb2 and anti-PLC-γ1 antibodies followed by blotting with anti-LAT. As shown in Fig. 5B, equal amounts of Grb2 or PLC-γ1 were immunoprecipitated from unstimulated or stimulated lysates. When the membrane was blotted with anti-phosphotyrosine antibody, a 36-38 kDa tyrosine phosphorylated protein was detected in association with Grb2 and PLC-γ1 only in activated T cells. This protein was identified as LAT by immunoblotting with anti-LAT antibody. CIP treatment of the immunoprecipitate enhanced LAT detection. p85 in anti-Grb2, but not in anti-PLC-γ1 immunoprecipitates in lysates of activated Jurkat cells, was also detected. There was also some low level of association between Grb2 and PLC-γ1 probably through LAT (data not shown).

TCR-mediated activation results in tyrosine phosphorylation of many substrates (Figs. 1A and 4C). To identify additional proteins, capable of binding LAT following T cell activation, anti-LAT antisera was used to immunoprecipitate LAT from lysates of resting and activated Jurkat cells. As shown in Fig. 5C, several tyrosine phosphorylated proteins with molecular weights of about 70, 76, 100, 120, 135 kD were specifically co-precipitated with anti-LAT only from lysates of stimulated Jurkat cells. By using a panel of antibodies to immunoblot known tyrosine kinase substrates, some of these proteins were identified as PLC-γ1 (135kD), Cbl (120 kD), Vav (100 kD) (not shown). The 76kD protein was most likely SLP-76. The associations of SLP-76 and Cbl with LAT were probably mediated

indirectly through Grb2, because both bind the Grb2 SH3 domain. Vav was likely detected because of its association with SLP-76. The 70 kDa protein was not identified, and was not Sam68 or ZAP-70. Anti-LAT immunoprecipitates from stimulated Jurkat also contained the p85 subunit of PI3K and Grb2 (not shown). The results in this example, demonstrate that LAT is present in signaling complexes containing multiple critical molecules.

EXAMPLE 7

In this example, the function of LAT in the TCR signal transduction pathway is shown. The hypothesis from the above studies is that the association of tyrosine phosphorylated LAT with the various signaling molecules is required for the TCR signal transduction process. To directly address the role of LAT in signaling through the TCR, wild type LAT or a mutant form of LAT was overexpressed and the effect of this overexpression examined on TCR-mediated activation. The mutant form of LAT employed in these experiments contains Phe for Tyr substitutions at both positions Y171 and Y191 (Y171/191F). These two tyrosine residues were chosen for mutagenesis because they are within identical YVNV motifs, a motif which has previously been shown to mediate the binding of Shc to Grb2 (See Songyang *et al.*, Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk and Vav. *Mol. Cell. Biol. 14*, 2777-2785, 1994). Moreover, phosphorylation at Tyr 191 was detected during microsequencing and mass spectrometry analysis of peptides from p36-38 (see above).

To investigate the effect of mutant LAT on protein-protein interactions, stable cell lines overexpressing myc-tagged wild type or Y171/191F LAT were established. Myc-tagged LAT was immunoprecipitated with anti-myc antibody from unstimulated or C305 stimulated transfectants. As shown in Fig. 6A, blotting with anti-LAT antibodies demonstrated that the amount of LAT immunoprecipitated with anti-myc was comparable in the two cell lines. The myc-tagged LAT appears as a doublet as does endogenous LAT. WT and Y171/191F LAT were both tyrosine phosphorylated upon stimulation, though the level of Y171/191F tyrosine phosphorylation was less than that of WT LAT, suggesting that tyrosine residues besides Y171 and Y191 are phosphorylated. Though p85, Grb2 and PLC-γ1 were observed to bind the WT form of LAT, mutations at Y171 and Y191 abolished the binding of Grb2 and p85, and greatly reduced the binding of PLC-γ1 upon activation. In addition, mutations at Y171 and Y191 prevented the association of SLP-76, Vav and Cbl, but the 70 kDa tyrosine-phosphorylated protein remained associated with LAT. Additionally, the Ras

activator protein SOS was observed to co-precipitate with LAT, likely due to its interaction with Grb2. The association with SOS was also disrupted by the double Y to F mutations.

The functional effect of mutant LAT overexpression was assayed by determining the effect on transcriptional activation of AP-1 and NF-AT, both involved in TCR-mediated transcriptional events. Jurkat TAg cells, were transiently transfected with wild-type LAT, mutant LAT(Y171/191F), or vector only, together with a reporter construct for secreted alkaline phosphatase (SEAP) driven by the AP-1 or NF-AT response elements. Fig 6B demonstrates that overexpression of WT LAT did not result in any significant effect on AP-1 transcriptional activity and only slightly increased NF-AT transcriptional activity compared with transfection of the control vector (see Fig 6). However, overexpression of the mutant form of LAT (Y171/191F) blocked TCR-mediated AP-1 and NF-AT transcriptional activity. The results in this example indicates that LAT plays an important role in activation of transcription mediated by AP-1 and NF-AT following TCR stimulation.

From the above, it should be clear that the present invention provides methods and compositions useful for identifying signaling pathway agonists and antagonists. The methods and compositions are, in this manner, useful for identifying compounds that may be used *in vitro* and *in vivo* in the context of T cell, NK cell and mast cell responses.

EXAMPLE 8

In this example the discovery of LAT palmitoylation sites and the use of LAT and LAT with modified palmitoylation sites in the TCR signal pathway is shown. Human and murine LAT contain two conserved cysteines, C26 and C29, near the punitive transmembrane domain. These features suggest that LAT could be a member of a class of transmembrane proteins modified by palmitate at juxamembrane cysteine residues. To examine whether LAT is palmitoylated, Jurkat cells were metabolically labeled with [^{3}H]palmitate. LAT was immunoprecipitated with anti-LAT antiserum, and immunoprecipitates were analyzed by SDS-PAGE and flourography. [^{3}H]palmitate was found to be incorporated into LAT. When an identical gel was treated with 1M hydroxylamine, the [^{3}H] signal was almost completely lost, indicating that the addition of palmitate to LAT was through S-acylation.

Many palmitoylated proteins, such as the Src family kinases Lck and Fyn, are targeted into glycolipid-enriched microdomains (GEMs) of the plasma membrane (Alland *et al.*"Dual myristylation and palmitoylation of Src family member p59^{fyn} affects subcellular localization" *J. Biol. Chem.* 269:16701-16705, 1994; Shenoy-Scaria *et al.*"Cysteine of Src

family protein tyrosine kinases determines palmitoylation and localization in caveolae" *J. Cell Biol.* 126:353-363, 1994). GEMs are operationally characterized by resistance to Triton extraction at 4°C and are found in low-density gradients of a sucrose gradient (Simons and Ikonen "Functional rafts in cell membranes" *Nature* 387:569-572, 1997). Since LAT is palmitoylated, we next examined whether LAT localizes to GEMs. Jurkat cells were solubilized with 1% Triton, and lysates were subject to overnight ultracentrifugation over a sucrose gradient (5%/30%/40% step gradient). Among a total of 12 fractions collected, fraction 3, at the interface between 5% sucrose and 30% sucrose, contained the low-density membrane fraction including the GEMs (Brown and Rose "Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface" *Cell* 68:533-544, 1992). Fractions 8-12 from the 40% sucrose section were the Triton-soluble fractions. LAT was detected by immunoblotting and was found in both GEM and Triton-soluble fractions. LAT in the GEM fractions (*e.g.* fraction 3) was predominately the p36 form. Two prominent forms of LAT, p36 and p38, were detected in Triton-soluble fractions (*e.g.* fraction 10). LAT was more abundant in fraction 3 than in fraction 10 as determined by immunoblot. When a sample of fraction 3 was diluted 16-fold, the amount of LAT still exceeded that found in the undiluted fraction 10. As controls for GEM purification, we immunoblotted the same membrane with anti-Lck and anti-ZAP70 antibodies. Lck was observed in both GEM and Triton-soluble fractions, but ZAP-70 was found only in Triton-soluble fractions, as expected.

To study the relationship between palmitoylation and GEM localization, Jurkat cells were labeled with [^{3}H]palmitate for 3 hr, lysed in 1% Triton, and subject to ultracentrifugation on a sucrose gradient. LAT was then immunoprecipitated from GEM and Triton-soluble fractions, resolved by SDS-PAGE, and analyzed by both fluorography and anti-LAT immunoblotting. The anti-LAT immunoblot showed that an approximately equal amount of LAT was precipitated from these two fractions. However, LAT in the GEM fraction was clearly more palmitoylated, indicating that palmitoylated LAT predominately localizes to GEMs.

Since LAT is present in both GEM and Triton-soluble fractions, we next examined in which fraction tyrosine phosphorylated LAT can be found after stimulation of Jurkat cells. We also studied whether activation of T cells induces redistribution of other signaling molecules into GEMs. Crosslinking with the anti-CD3ε antibody, OKT3, leads to tyrosine phosphorylation of many cellular proteins and activation of T cells. Unstimulated or OKT3-

stimulated Jurkat lysates were subjected to sucrose gradient centrifugation to separate GEM fractions from Triton-soluble fractions. Each fraction was analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody and antibodies against each individual protein. As shown in Fig. 13A, LAT and Lck were basally tyrosine phosphorylated in both GEM and Triton-soluble fractions from unstimulated cells. A tyrosine phosphorylated 80 kD diffuse band was also observed in the GEM fractions. LAT phosphorylation results in interactions with Vav, a membrane associated guanine nucleotide releasing factor. While the majority of Vav localized to the Triton-soluble fractions, some tyrosine phosphorylated Vav was detected in the GEM fractions (Fig.13A) and fractions 5-7, as shown by anti-Vav blot in Fig.13C.

Upon stimulation with OKT3, most tyrosine phosphorylated LAT appeared in the GEM fractions, although some was in the Triton-soluble fractions (Fig. 13B). There was no obvious change in Lck tyrosine phosphorylation, but the 60 kD form of Lck, well known to accompany TCR activation (Marth *et al.*"Lymphocyte activation provokes modification of a lymphocyte-specific protein tyrosine kinase (p56*lck*)" *J. Immunol.* 142:2430-2437, 1989), was increased in Triton-soluble fractions upon stimulation, as shown by the anti-Lck blot (Fig. 13C). Other prominent tyrosine phosphorylated proteins present in the GEM fractions upon stimulation were PLC-γ1 and Vav (Fig. 13B). Blotting with antibodies to particular signaling molecules supported the conclusion that certain molecules re-distribute to GEMs upon TCR activation (Fig. 13C). The amount of PLC-γ1 increased in the GEM fractions and fractions 5-7 upon stimulation. Like PLC-γ1, the amount of Vav in those fractions also increased after stimulation. The distribution of the p85 subunit of PI-3 kinase was similar to Vav. There was also some increase of Grb2 in the GEM fractions after stimulation (Fig. 13C). Cbl and ZAP-70 were present in Triton-soluble fractions, but they did not re-distribute into GEMs after stimulation (Fig.13C). We used the detergent octyl-glucoside to solubilize GEMs and Triton-soluble fractions in order to assess LAT-associated proteins in the two pools. This detergent is capable of solubilizing Triton-insoluble GEMs (Brown and Rose "Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface" *Cell* 68:533-544, 1992). Following extraction with this detergent, we observed by co-immunoprecipitation that more PLC-γ1 and Vav associated with LAT in GEMs than LAT in Triton-soluble fractions, suggesting that LAT in GEMs interacts with these signaling molecules following activation.

. The present invention contemplates assays for the screening of compounds that are agonistic or antagonistic for LAT activation or that are agonistic or antagonistic for LAT binding of associated molecules. For example, but not by way of limitation, it is contemplated that compounds will be screened using LAT, or portions thereof, and the methods disclosed herein *(e.g.* determining the extent of LAT incorporation into GEMs). In such an assay compounds that activate LAT would result in a greater amount of LAT being associated with GEMs, as compared to controls. Likewise, compounds that interfered with LAT activation would result in lesser amounts of LAT being associated with GEMs, as compared to controls. Additionally, but not by way of limitation, it is contemplated that compounds that modulate LAT binding to associated molecules (for example, but not limited to, Vav, PLC-γ1, Grb2, Sos, cb1, SLP-76, SLAP, and PI-3 kinase) will be screened using LAT, or portions thereof, and the methods disclosed herein. Modulation of LAT binding of any of these molecules can be determined by methods known to those practiced in the art (*e.g.* immunoblot, measurement of binding coefficients, measurement of downstream activation events).

To test whether two cysteines, C26 and C29, in human LAT are the sites for addition of the palmitate moiety, we made myc-tagged LAT mutants with single cysteine to alanine mutations (C26A, C29A) and a double mutant (C26/29A). We also made a mutant LAT (Δtm) with a deletion of the N-terminal region (residues 1-22) including the transmembrane domain, to investigate the role of the transmembrane domain in LAT palmitoylation and function. T-antigen transformed human kidney fibroblasts (293T cells) were transiently transfected with these constructs and metabolically labeled with [^{3}H]-palmitate. LAT was then immunoprecipitated with anti-myc antibody from lysates of these transfected cells. As seen in Fig.14 (top panel), two forms of LAT labeled with [^{3}H]-palmitate were detected in immunoprecipitates of wild type (wt) LAT. For the C26A mutant, only the p38 form was labeled with [^{3}H]-palmitate and the amount of ^{3}H label in C26A was less than in wt. Two forms of LAT labeled with [^{3}H]-palmitate were also observed with the C29A mutant, but compared with wt, the amount of ^{3}H incorporated into the p38 form was reduced and ^{3}H label in the p36 form was barely detectable. A similar trace amount of ^{3}H was present in the LAT double cysteine mutant (C26/29A) and in the transmembrane domain deletion mutant (Δtm).

The same samples from the above labeling experiment were loaded on another gel and analyzed by blotting with anti-myc antibody. As shown in Fig.14 (lower panel), similar

amounts of proteins were expressed in each lane. Note that the only the p314 form of LAT was detected by blotting indicating in transient transfections the p36 form was rare. These results indicate that C26 and C29 are the major sites for LAT palmitoylation. The trace amount of ^{3}H in C26/29A and Δtm mutant LAT is most likely due to metabolic conversion of [^{3}H]-palmitate into [^{3}H]-amino acids, although we could not totally exclude the possibility that LAT might be palmitoylated at a low level at other site(s).

Because mutations of two cysteines, C26 and C29, affected the palmitoylation of LAT, we next tested whether these mutations also altered membrane localization and the partition of LAT into GEMs. We examined the membrane localization of wt and mutant LAT by immunofluorescence staining of transiently transfected 293T cells. There was no significant difference in localization between wt LAT and any of the cysteine mutants. However, deletion of the transmembrane domain of LAT resulted into a predominantly cytosolic localization (Fig. 15A). We also transiently transfected Jurkat/TAg cells (SV40 large T antigen transformed Jurkat cells, Clipstone and Crabtree "Identification of calcineurin as a key signaling enzyme in T-lymphocyte activation" *Nature* 357:695-697, 1992) with LAT constructs and analyzed the subcellular distribution of LAT by fractionation of transfected cells into cytosolic and particulate fractions. Myc-tagged wt LAT, C26A, C29A, and C26/29A mutants predominantly localized to the particulate fraction, while the LAT Δtm was detected mainly in the cytosolic fraction (Fig. 15B).

Although C26A, C29A, and C26/29A LAT mutants all localized to the plasma membrane, it was possible that these mutations might affect the targeting of LAT into GEMs. Transiently transfected Jurkat cells were lysed in 1% Triton, and GEM fractions were purified using a sucrose gradient. Samples from fractions 3 and 10, as representatives of the GEM and Triton-soluble fractions, respectively, were analyzed on SDS-PAGE and immunoblotted with anti-myc antibody. The wt LAT and all of the LAT mutants were expressed equally in Triton-soluble fractions (Fig. 15C). Two forms of wt LAT and C29A were observed in these fractions, though the p36 form of C29A was barely detectable. Strikingly, only the wt LAT and a small amount of C29A were present in the GEM fractions. The plasma membrane localization of C26A and C26/29A and the palmitoylation of the C26A mutant were not sufficient for GEM targeting. The cytosolic LAT Δtm mutant was not seen in the GEM fraction, as expected. These results indicate that, while the transmembrane domain alone suffices for LAT plasma membrane localization, palmitoylation

at C26 is essential for GEM targeting. Palmitoylation only at C29 is not sufficient for GEM targeting, but it does increase the efficiency of C26-mediated targeting.

We next addressed whether palmitoylation and targeting into GEMs were necessary for the phosphorylation of LAT by tyrosine kinases. Jurkat cells were transfected with mutant LAT constructs and stimulated with OKT3 at 36 hours after transfection. The p36 form of wt myc-tagged LAT was rapidly tyrosine phosphorylated upon OKT3 stimulation (Fig.10). The p36 form of the C29A mutant was also tyrosine phosphorylated, though less than wt. Only very weak tyrosine phosphorylation of the p314 form of C26A or C26/29A was observed. Surprisingly, LAT Δtm was also tyrosine phosphorylated to some extent. This could be explained by the presence of activated ZAP-70 in the cytosol following stimulation. These activated ZAP-70 molecules might phosphorylate LAT Δtm in the cytosol. Endogenous LAT, however, must be in GEMs to become optimally tyrosine phosphorylated during activation. Blotting the same membrane with anti-myc antibody showed that the amount of LAT was nearly equal in all lanes. This protein blot also revealed the presence of a band at 140 kD in the wt and C29A lanes. This band could be detected with anti-LAT antibody. This 140 kD band could be a dimer of LAT, resistant to treatment with SDS and DTT. In conclusion, our results clearly show that LAT palmitoylation at C26 and C29 not only is required for LAT palmitoylation, but also is necessary for efficient tyrosine phosphorylation by tyrosine kinases during T cell activation.

The present invention contemplates the use of these LAT mutations (C26A, C29A, C26/29A and Δtm), or parts thereof, in screening assays for compounds that are agonistic or antagonistic to LAT activation. Additionally, the present invention contemplates the use of these LAT mutations in screening assays for compounds that will allow for the activation of T cells in the presence of non-functional (*e.g.* non-palmitoylated) LAT.

EXAMPLE 9

In this example the staining of both cryostat and paraffin embedded normal tissues and cells was performed. Positive staining for LAT was crisp and mainly localized on the cell membrane and the sub-plasmalemmal area. Variable number of cells also showed diffuse cytoplasmic reactivity and, on occasion, dot-like positivity in the Golgi region. On paraffin sections, optimal staining was observed in tissues fixed in both buffered formalin or B5, whereas occasionally some background staining was observed for tissues fixed in Bouin or

Hollande. Decalcification of bone marrow specimens in EDTA did not affect LAT immunoreactivity.

In the thymus, expression of LAT was identifiable throughout all stages of thymocyte differentiation, including the large cortical blasts. In embryos of 10-12 weeks of gestation, thymuses already showing a lobular architecture contained lymphoid cells that expressed LAT and CD3, but were negative for TdT indicating that LAT expression precedes TCR rearrangement.

In peripheral lymphoid tissues, LAT-positive lymphocytes were located in the known T cell areas in lymph nodes and spleen, and the immunoreactivity paralleled that obtained with anti-CD3 in both frozen and paraffin sections. In the small intestine, intraepithelial T cells were also positive for LAT. In bone marrow, LAT was expressed by the sparse T lymphocytes present in interstitial spaces, and also by platelets and megakaryocytes, that exhibited a strong reactivity in the cytoplasm; all other hematopoietic cells were negative. Reactivity for LAT was also noticed on tissue mast cells, in the form of delicate plasma membrane and granular cytoplasmic labeling. In all tissues, non-T cell components, including B cells, macrophages, plasmacytoid monocytes, epithelioid histiocytes and dendritic cells, were completely negative for LAT. Similarly, LAT was not expressed on endothelial cells and epithelia, including thymic epithelium and Hassal bodies.

On peripheral blood mononuclear cells (PBMC), LAT was expressed, in addition to CD3+ T cells, on resting CD16+ and CD56+ NK cells. LAT staining was also found on T and NK cells in culture with rIL-2; on CD3-CD16+CD56+ NK cells purified from PBMC, LAT was obviously expressed, although the intensity of reactivity was much weaker than that recognized on purified CD3+ T cells.

EXAMPLE 10

In this example the staining of LAT in neoplastic tissues was performed. Neoplastic cells in all cases of Hodgkin's (15 cases) and B cell non-Hodgkin's lymphomas (100 cases) (see Table 1) were negative for LAT, but contained variable amounts of LAT-reactive non neoplastic T cells. In T-cell lymphomas (excluding anaplastic large cell lymphomas (ALCL)), LAT stained 14/14 lymphoblastic lymphomas, 42/43 peripheral T cell lymphomas, and 11/12 NK/T cell lymphomas. The intensity of reactivity and the percentage of labeled cells varied among different types of T cell lymphomas. In lymphoblastic lymphomas all neoplastic cells were strongly labeled by anti-LAT; in contrast, in peripheral T cell and NK/T

cell lymphomas the reactivity was intense in 214/53 (52.14%) cases, and it was recognizable in the vast majority of cells in 50/53 (94.3%). LAT expression showed no substantial differences in peripheral T cell lymphomas with relationship to primary location, cell morphology, and immunophenotype, with the exception of NK/T cell lymphomas, which were more frequently characterized by weaker and a lower number of labeled cells.

Table 1.

Details of negative LAT staining Hodgkin's lymphomas and malignant B cell lymphomas analyzed.

	N° of cases
Hodgkin's Lymphomas	
Lymphocyte predominance	2
Nodular sclerosis	10
Mixed cellularity	2
Total	**15**
B-cell lymphomas	
I. Precursor B-lymphoblastic leukemia/lymphoma	8
II. Peripheral B-cell neoplasms	
B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma	14*
Lymphoplasmacytoid lymphoma	3
Mantle cell lymphoma	11**
Follicle center cell lymphoma, follicular	16
Marginal zone B-cell lymphoma	
nodal	1
extranodal	4
Hairy cell leukemia	6
Myeloma	8
Diffuse large B-cell lymphoma	25***
Burkitt's lymphoma	4
Total	**100**

*: one with immunoblastic transformation; ** one "blastoid" variant; ***: included four T-cell rich B large cell lymphomas and three CD30+/ALK1- anaplastic large cell lymphomas.

The ALCL included 39 extra-cutaneous and 5 purely cutaneous cases; on the basis of the expression of at least one T cell marker (CD3, CD5, CD14, CD43, CD45R0, TCR-β) and no B cell markers (CD20 or CD79a), 25 cases were classified as T cell and 19 cases as null-cell phenotype. 14 cases (31.14%) reacted with LAT; all were represented by extra-cutaneous lymphomas, ten of them were of T cell phenotype, while four were null-cell. LAT positive ALCL showed intense reactivity in 5/14 (35.7%) cases, and it was recognizable in the vast majority of cells in 10/14 (71.4%).

LAT and CD3 expression was compared in T cell and NK/T cell lymphomas (excluding ALCL). In T-lymphoblastic lymphoma, LAT positivity was more intense and diffuse than CD3. Among peripheral T cell lymphomas, only two LAT-negative cases were observed. One was a large cell lymphoma presenting an aberrant phenotype with loss of CD3,CD5, CD43 and TCR-β, the other LAT negative case was a NK/T which strongly expressed cytoplasmic CD3. Three cases of LAT positive cutaneous T-cell lymphomas did not express CD3 (two also lacked CD5), but were positive for CD2, CD4 and TCR-β.

A comparison was made regarding LAT, CD3 and ALK1 expression in ALCL. The distribution of LAT, CD3 and ALK1 in ALCL is reported in Table 2. Seven ALCL cases were LAT+CD3+, 7 were LAT+ CD3-, 9 were LAT- CD3+, and 21 were LAT-CD3-. Among ALCL T cell type (25 cases), CD3 labeled a higher number of cases (19/25; 76%) than LAT (10/25; 40%). Interestingly, LAT and CD3 expression in ALCL T cell type was discordant in a significantly higher number of cases than in other peripheral T cell lymphomas (12/25 versus 4/55, respectively; Fisher's exact test: $p<0.001$). Immunostaining for ALK1 was available in 42 cases and showed nuclear ± cytoplasmic positivity in 10. Although the majority of ALK1+ cases were LAT+ (6/10), in comparison with ALK1- cases (14/32), this difference was not statistically significant (Fisher's exact test: $p=0.059$).

Expression of LAT was also examined in Hematopoietic, non-lymphoid neoplasms. In chronic myeloproliferative disorders and myelodysplastic syndromes, cytoplasmic LAT expression was consistently identified in normal and atypical megakaryocytes, including the micro-megakaryocytes typically found in chronic myeloid leukemia and myelodysplasia. Among 214 cases of acute non-lymphoid leukemia, the only positive cases were represented by two megakaryocytic leukemias, where the blast cells were labeled in their cytoplasm; an additional case of chronic myeloid leukemia in blastic transformation contained two cell populations, one formed by myeloid blasts (myeloperoxidase and CD34 positive), another by megakaryocytic blasts, expressing LAT, Factor VIII-related antigen and CD34. In all

Table 8. Details of LAT, CD3 and ALK1 staining in anaplastic large cell lymphomas (ALCL).

	T-cell type				Null-cell type			
	LAT+ CD3+	LAT+ CD3-	LAT- CD3-	LAT- CD3+	LAT+ CD3+	LAT+ CD3-	LAT- CD3-	LAT- CD3+
ALK1 positive	3	3	1				3	
ALK1 negative	4		4	8		4	12	
ALK1 n.a.*			1	1				
Total	7	3	6	9	0	4	15	0

*: ALK1 not assessable because only B5-fixed specimens available and poor reactivity of anti-ALK1 on this fixative.

pathological conditions, reactivity of LAT on megakaryocytes was strong and particularly helpful in the identification of abnormal forms; moreover, it was frequently stronger than that of other megakaryocytic markers on paraffin sections, such as Factor-VIII related antigen and CD61. 4/5 cases of mastocytosis showed LAT positivity, including 3 of 3 cases of cutaneous mastocytosis, and 1 of 2 cases of systemic mastocytosis, as with normal mast cells, LAT expression in neoplastic mast cells was finely granular in the cytoplasm and delicate on the cell membrane, a pattern of staining that clearly differed from that on T cells.